University of Alberta

Cold shock response and adaptation in *Anabaena* sp. strain PCC 7120

by

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

in

Microbiology and Biotechnology

Department of Biological Sciences

Edmonton, Alberta

Fall 2005

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DEDICATION

To my parents, my wife, and my children, Tala, Ayah, and Omar

ABSTRACT

The work described in this thesis is focused on advancing our understanding of cold shock response and adaptation in cyanobacteria. For this purpose two approaches were employed; (I) Investigating the role of a cold shock regulated RNA helicase, CrhC, in cold shock adaptation, (II) Identifying downstream protein target(s) in the cold shock signal transduction pathway.

The role of CrhC from the cyanobacterium *Anabaena* sp. strain PCC 7120 during cold shock was investigated by identifying its location within cells and by identifying proteins associated with CrhC. CrhC was found by cell and membrane fractionation and immunogold labeling to be plasma membrane associated, exhibiting polar-biased localization and co-localization with the ribosome. Furthermore, the proteins co-purifying with CrhC by co-immunoprecipitation are also plasma and outer membrane associated. These findings suggest that CrhC is involved in the transertion of cold shock induced gene products into or across the plasma membrane .

Interestingly in contrast to other organisms, the *Anabaena* genome has two ribosomal protein S1 genes encoding Rsp1A and Rps1B. I have found that one of the two ribosomal S1 proteins, Rps1B (33 kDa), is cold induced, which may contribute to the cold shock adaptation of the ribosome. While investigating downstream targets in the cold shock signal-transduction pathway in *Anabaena*, other ribosome related modifications were also observed. *In vitro* phosphorylation and immobilized metal affinity chromatography (IMAC) displayed a consistent

increase in the phosphorylation of two proteins within 0.25 h of cold shock. The proteins were identified as translation elongation factor (EF-Tu) and ribosomal protein S2 (Rps2). Phosphorylation of EF-Tu would prevent ternary complex formation inhibiting protein translation at the elongation step. Rps2 is necessary for recruitment of ribosomal protein S1 required for translation initiation. Hyperphosphorylation of Rps2 may result in a conformational change hindering S1 binding, resulting in a block in translation initiation. In conclusion, cold shock induced modifications occurring in the ribosome including increased expression of Rps1B, and the hyperphosphorylation of EF-Tu and Rps2 may contribute to the formation of a cold adapted ribosome.

Based on the results of this thesis, a model of cold shock response and adaptation in *Anabaena* is proposed.

ACKNOWLEDGMENTS

I would like to express my sincere appreciation and gratitude to the following people who have made the completion of this thesis possible:

Dr. G. Owttrim for his guidance, advice, patience, and friendship and for being a great supervisor.

My supervisory committee, Dr. G. Goss, and Dr. N. Adames for their valuable suggestions and help.

Dr. D. Chamot for her advice, support, and for being a great person to work with.

Wendy Magee who cloned, and sequenced *crhC*.

Esther Yu for generating anti-CrhC antibodies, and performing the biochemical characterization of CrhC.

Dr. Sonya Kujat-Choy for helpful suggestions and support.

Dr. L. Frost, Dr. W. Page, Dr. T. Raivio, and Dr. B. Leskiw for their advice, support and the use of lab equipment and reagents.

Jessica Brown and Laura Patterson-Fortin for being great colleagues.

Biological Sciences Microscopy service unit members, Rakesh Bhatnagar, Randy Mandryk, and Jack Scott for their advice and support in producing the electron microscopy images for this study.

Microbiology RIG support staff for their help and support.

Finally, I want to thank my wife, Ahlam, for her love, encouragement, and emotional support.

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LIST OF ABBREVIATIONS

APS	ammonium persulfate
ATP	adenosine 5`-triphosphate
bp	base pair(s)
°C	degrees centigrade
CRD	cross reacting determinant
CSP	cold shock protein
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylene diamine tetra acetic acid
FA	fatty acid
g	gram(s)
x *g*	gravitational force
HEPES	N-[2-hydroxyethyl]piperazine-N`-[2-ethanesulfonic acid]
h	hour(s)
IPTG	isopropyl-β-D- thiogalactoside
kDa	kilodalton(s)
L	liter(s)
M	molar
MALDI	Matrix assisted laser desorption/ionisation
mg	milligram(s)
min	minute(s)
ml	milliliter(s)

mM	milimole(s)
nm	nanometer(s)
OD	optical density
PBS	phosphate buffered saline
PMSF	phenylmethylsulfonyl fluoride
%	percent
pmol	picomole(s)
PPiB	peptidyl-prolyl cis-trans isomerase
Rbp	RNA binding protein
RBS	Ribosome binding site
RNA	ribonucleic acid
RNase	ribonuclease
DNase	deoxyribonuclease
rpm	revolutions per minute
sarkosyl	N-laurylsarcosine
SDS	sodium dodecyl sulfate
sec	second(s)
TAP	tandem affinity purification
TBS	Tris buffered saline
TF	trigger factor
TOF	Time of flight
TRP	transient receptor potential
µg	microgram(s)

µL	microliter(s)
UTR	untranslated region
UV	ultraviolet
V	volt(s)
v/v	volume per volume
W	Watt(s)
w/v	weight per volume

Chapter 1

Introduction

1.1 General physiological effects of cold shock

Temperature change is one of the most common stresses microorganisms encounter in nature. While the adaptive response for a sudden upshift in growth temperature (heat shock) has been well-studied, the adaptation to a sudden downshift in growth temperature (cold shock) is not well understood. Cold shock affects numerous essential functions of the cell, most importantly it results in decreases in protein synthesis and membrane fluidity (Thieringer *et al.*, 1998; Yamanaka, 1999: Golovlev, 2003; Gualerzi *et al.*, 2003; Weber and Marahiel, 2003; Phadtare, 2004). Therefore, it is not surprising that microorganisms possess sophisticated mechanisms of response and adaptation to the life threatening influence of low temperature. Cellular adaptation to cold shock involves re-regulation of gene expression, resulting in alterations to gene and protein expression and the *de novo* synthesis of a specific set of proteins known as the cold shock proteins (CSPs) (Thieringer *et al.*, 1998; Yamanaka, 1999: Golovlev, 2003; Gualerzi *et al.*, 2003; Weber and Marahiel, 2003; Phadtare, 2004). This coordinated regulation of cold shock induced gene expression and the resulting pattern of *de novo* CSP synthesis constitutes the cellular response and subsequent adaptation to cold shock. An efficient cold shock response would require a mechanism to selectively translate CSP mRNA, producing protein products required for adaptation to the reduced temperature.

Our current knowledge about the effect of cold shock on metabolism dates to the late 1960's and was initially examined in *Escherichia coli* both *in vivo* (Anderson 1975; Chlamovitch and Anderson 1972; Das and Goldstein. 1968;

Friedman *et al.*, 1969b and 1971) and *in vitro* (Friedman *et al.*, 1969a; Szer 1970). Friedman and coworkers (1971) demonstrated *in vitro* that synthesis of f-2 viral coat protein proceeded to completion, but f-2 mRNA translation was not initiated after cold shock. The interpretation was that cold shock results in a block in protein synthesis at the level of translation initiation. Subsequently, Pope *et al.*, (1975), observed that, at a low temperature (5°C), translation elongation was also reduced, decreasing by 90 to 95% compared to that observed at normal growth temperature (37°C). These findings suggested that protein synthesis is regulated at the level of both translation initiation and elongation during cold shock. The mechanism by which these processes are regulated by cold shock are just now starting to be elucidated.

1.2 Cold shock response in bacteria

1.2.1 Bacterial cold shock proteins and their relative functions in *E. coli* and *Bacillus subtilis*

Cold shock has traditionally been defined as a sudden downshift from the normal growth temperature by a minimum of 10°C (Inouye 1999). Several CSPs have been identified in bacteria, with cold shock adaptation being most extensively studied in *E. coli* and *Bacillus subtilis* (Golovlev, 2003; Gualerzi *et al.*, 2003; Weber and Marahiel, 2003; Phadtare, 2004). CSPs are categorized into two classes: class I proteins are expressed at an extremely low level at normal growth temperature and are dramatically induced to high levels (>10 fold) after the temperature downshift; and class II proteins are present at normal growth

temperatures and are moderately induced after cold shock (<10 fold) (Thieringer *et al.*, 1998). The classic example of a class I CSP is the major cold shock protein A (CspA). CspA was first identified in *E. coli*, and subsequently homologs were identified in more than 60 bacterial species (Phadtare *et al.*, 2000). CspA is a nucleic acid binding protein that belongs to a family of low molecular weight CSPs (7 kDa), whose members represent the majority of CSPs known to date (Goldstein *et al.*, 1990; Graumann *et al.*, 1996; Phadtare and Inouye, 1999; Phadtare *et al.*, 2003). Other examples of class I proteins include CspB (Lee *et al.*, 1994), CspG (Nakashima *et al.*, 1996), CspI (Wang *et al.*, 1999), an RNA helicase CsdA (DeaD) (Toone *et al.*, 1991), the ribosomal binding protein RbfA (Dammel and Noller, 1995), NusA (Friedman *et al.*, 1984), and PNPase (Donovan and Kushner, 1986). Examples of class II proteins include RecA (Walker, 1984), translation initiation factor-2 (IF2) (Gualerzi and Pon, 1990), IF1, IF3 (Giuliodori *et al.*, 2004), H-NS (Dersch *et al.*, 1994), the α subunit of DNA gyrase (Sugino *et al.*, 1977), Hsc66, HscB (Lelivelt and Kawula, 1995), trigger factor (TF) (Kandror and Goldberg, 1997), pyruvate dehydrogenase, and dihydrolipamide transferase (Jones and Inouye, 1994). A more extensive, although not exhaustive list of CSPs with their relative functions is presented in Table 1.1. As described earlier, the major effects of cold shock are on protein synthesis and membrane fluidity, thus, the role for the majority of CSPs in regards to cold shock adaptation involves the alleviation of these constraints (Thieringer *et al.*, 1998; Yamanaka, 1999: Golovlev, 2003; Gualerzi *et al.*, 2003; Weber and Marahiel, 2003; Phadtare, 2004).

Table 1.1. Proposed function of cold shock proteins from *E. coli* and *B. subtilis* (list is adapted from Phadtare *et al.*, 2000; Cairrão *et al.*, 2003; Gualerzi *et al.*, 2003)

Protein(s)	Function
E. coli	
CspA, CspB, CspE, CspG, CspI	RNA/DNA chaperones
CsdA	RNA unwinding/ribosome assembly
RbfA	Ribosomal binding factor
NusA	Transcriptional termination and antitermination
PNPase	3`-5` exonuclease; degradosome component
RNase R	Maturation of SsrA/tmRNA
RecA	DNA recombination/DNA repair
IF1	Translation initiation
IF2	Translation initiation factor
IF3	Translation initiation
pY	Translation (A-site) inhibitor
H-NS	Structuring chromosomal DNA
HUβ	DNA supercoiling
DnaA	DNA binding and replication
α-subunit of DNA gyrase	DNA supercoiling
Hsc66	DnaK homologue
HscB	DnaJ homologue
Trigger factor (TF)	Protein folding
Dihydrolipoamide transferase	Energy generation
Pyruvate dehydrogenase	Energy generation
OtsA	Trehalose phosphate synthase
OtsB	Trehalose phosphatase
B. subtilis	
CspB, CspC, and CspD	RNA chaperones
CheY	Chemotaxis
Hpr	Sugar uptake
Ribosomal protein S6, andL7/L12	Translation
Peptidyl prolyl *cis/trans* isomerase	Protein folding
Cysteine synthase	General metabolism
Ketol-acid reductoisomerase	General metabolism
Glyceraldehyde dehydrogenase	General metabolism
Triosephosphate isomerase	General metabolism

1.2.2. CSPs role in protein synthesis

During the cold shock response there is a significant reduction in general protein synthesis, accompanied by a decrease in polysomes and a corresponding increase in the accumulation of 70S, 50S, and 30S monosomes (Das and Goldstein, 1968; Friedman *et al.*, 1969a; Broeze *et al.*, 1978; Farewell and Neidhardt, 1998; Yamanaka, 1999; Bayles *et al.*, 2000). These findings indicate that the major metabolic effect of cold shock involves alterations in translation. As described earlier it is now widely accepted that a major contributor to this block occurs at the point of translation initiation (Das and Goldstein, 1968; Friedman *et al.*, 1969b and 1971; Thieringer *et al.*, 1998; Graumann and Marahiel, 1999; Yamanaka, 1999: Golovlev, 2003; Gualerzi *et al.*, 2003; Weber and Marahiel, 2003; Phadtare, 2004). In apparent contradiction to this statement, CSPs are specifically synthesized in response to cold shock. It is quite evident that cold shocked cells possess the mechanisms that render them capable of reducing translation in general while allowing the selective translation of mRNAs coding for CSPs. However, they must also be capable of resuming translation of essential non-cold shock-induced mRNA in order to resume normal cellular function and growth during prolonged cold shock (i.e. adaptation to cold shock conditions). The fact that a significant number of CSPs are translation initiation factors (IF1, IF2, and IF3) and ribosome associated factors (RbfA, CspA, CspB and Dead/CsdA) indicate that the ribosome itself is a primary target of the cellular response to cold shock (Gualerzi and Pon, 1990; Gualerzi *et al.*, 2003; Giuliodori *et al.*, 2004).

Translation initiation in bacteria involves the interaction of the 30S ribosome subunit with the Shine-Dalgarno sequence, that is complementary to the 3′ end of the 16S RNA. The efficiency of translation initiation and regulation have been shown in different systems to correlate with the complexity of secondary or tertiary structures sequestering the Shine-Dalgarno sequence at the 5′ untranslated region (UTR) (Graumann and Marahiel, 1999). The primary step in translation initiation involves an interaction between mRNA and the 30S subunit which requires ribosomal protein S1 (Rps1)-mRNA complex interaction with the 16S RNA. For this step to occur both RNAs must be in a single stranded form (Schlax and Worhunsky, 2003). Non-cold shock mRNAs have short 5′ UTRs, generally less than 15 nucleotides long. In contrast, mRNAs encoding CSPs are characterized by 5′ UTRs longer than 100 nucleotides that fold into complex 3-D configurations (Thieringer *et al.*, 1998). The mechanism by which selective translation of mRNA is accomplished during cold shock is not known. However, it is believed that the secondary structure must be converted to a single stranded form for translation initiation to occur (Hurme and Rhen, 1998; Lim *et al.*, 2000; Schlax and Worthunsky, 2003). It is well established that duplex RNA in the 5′ UTR reduces or even blocks translation initiation (Graumann and Marahiel 1999; Schlax and Worthunsky, 2003).

In Gram-negative bacteria, under normal growth temperatures, ribosomal protein S1 (Rps1) appears to be essential for translation initiation of all mRNAs with short 5′ UTRs (Szer *et al.*, 1976; van Dieijen *et al.*, 1976) and for translation initiation complex formation at internal ribosome binding sites (rbs) (Tedin *et al.*, 1997, Schlax and Worthunsky, 2003). Rps1 binds transiently with the 30S subunit, an interaction which absolutely requires ribosomal protein S2 (Rps2) (Boni *et al.*, 1982 and 1991; Wimberley *et al.*, 2000). It has been suggested that the role of Rps1 in translation initiation is to assist positioning of the 30S subunit in close proximity to the translational start site by destabilizing secondary structure in an ATP-independent manner (de Smit and van Duin, 1994). However, the block in translation initiation during cold shock suggests that Rps1 unwinding activity may be insufficient to destabilize the thermodynamically stabilized long, highly structured 5′ UTRs of cold shock mRNA (Lu *et al.*, 1999). Therefore it has been proposed that active unwinding or destabilizing of secondary structures in 5′ UTRs will alleviate the block in translation initiation, thereby facilitating protein synthesis during cold shock (Thieringer *et al.*, 1998; Graumann and Marahiel, 1999; Yamanaka, 1999; Golovlev, 2003; Gualerzi *et al.*, 2003; Weber and Marhiel, 2003; Phadtare, 2004). Among the potential candidates to alleviate this block in translation initiation are the RNA helicase(s).

RNA helicases actively unwind duplex RNA using energy derived from nucleotide triphosphate hydrolysis (Fuller-Pace, 1994). A ribosome associated cold-induced RNA helicase, known as cold shock DeaD protein A (CsdA) (Jones *et al.*, 1996) known originally as DeaD (Toone *et al.*, 1991) has been identified in

E. coli. CsdA has been proposed to be involved in a variety of cellular processes including ribosome biogenesis (Charollais *et al*., 2004), mRNA degradation (Prud'homme-Généreux *et al*., 2004), and translation initiation (Jones *et al*., 1996; Toone *et al*., 1991; Lu *et al*., 1999; Moll *et al*., 2002). In support of its role during cold shock, the deletion of *csdA* results in an impairment of protein synthesis and cell growth under cold shock conditions linking its role in protein synthesis to cold shock adaptation (Jones *et al*., 1996). In addition, association of CsdA with the ribosome during cold shock suggested that CsdA function involves the unwinding of secondary structures at cold shock mRNA 5′ UTRs in an ATP-independent manner (Lu *et al*., 1999; Moll *et al*., 2002). However, the work of Charollais *et al*. (2004) has recently reported that *csdA* deletion results in a deficit of 50S ribosomal units and the accumulation of misassembled 40S large subunits at low temperature. These results suggest that CsdA is involved in the biogenesis of the 50S ribosomal subunit during cold shock.

Initially *csdA* was identified as a multi-copy suppressor of an *E. coli* temperature-sensitive mutation in the *rpsB* which encodes Rps2, where Rps1 and Rps2 expression was reduced at non permissive temperature (Toone *et al*., 1991). The lack of Rps2 reduces the recruitment of Rps1 to the 30S ribosomal subunit resulting in a reduction in protein synthesis specifically a reduction in translation initiation. These findings suggest that CsdA may compensate the Rps1 deficiency by destabilizing RNA secondary structures, thus facilitate translation initiation (Toone *et al*., 1991). The work of Moll *et al*. (2002) supports the conclusions of Toone *et al*. (1991), where they have shown that expression of plasmid borne

csdA restores both Rps1 and Rps2 on the ribosome in an *rspB*^{ts} at non-permissive temperature. These data position CsdA at the translation initiation step during cold shock. In support of this, recently, utilizing tandem affinity purification (TAP), several 30S subunit proteins were co-purified with CsdA including Rps1 and Rps2 (Butland *et al.*, 2005).

A second *E. coli* ribosome associated CSP, RbfA, is also bound to the 30S subunit of the ribosome rather than the 70S or polysomes (Dammel and Noller, 1995). RbfA overexpression resulted in a significant enhancement in the rate of adaptation to cold shock as compared to wild type cells (Jones and Inouye, 1996). Also, it has been reported that Class II cold shock proteins were constitutively expressed in *rpfA*$^-$ cells delaying adaptation of the protein synthesis machinery to cold shock, suggesting a role for RbfA in translation initiation and/or ribosomal biogenesis in response to cold shock (Jones and Inouye, 1996).

The most extensively studied CSPs are nucleic acid binding proteins CspA from *E. coli* and CspB from *B. subtilis*, which possess highly conserved RNA binding motifs (Schindelin *et al.*, 1993; Jones and Inouye 1994). Since CspA-related proteins bind RNA, it has been proposed that these proteins act as RNA chaperones, potentially destabilizing or minimizing mRNA secondary structure formation during cold shock, thereby facilitating translation initiation (Graumann *et al.*, 1997; Jiang *et al.*, 1997). The initiation step in protein synthesis requires the translation initiation factor, IF1, which binds directly to the 30S subunit of the ribosome (Carter *et al.*, 2001). Recently, on the basis of the fact that CspA and IF1 share a highly similar fold, heterologous expression of translation initiation

factor IF1 from *E. coli* in a *B. subtilis cspB / cspC* double deletion strain was shown to complement both the growth and sporulation defects observed for this mutant. These findings suggest a potential connection between CSPs and translation initiation during cold shock adaptation (Weber *et al.*, 2001). Furthermore, CspB co-localizes with ribosomes; however, this localization is only observed upon active transcription in *B. subtilis* during cold shock, suggesting CspB involvement in facilitating translation only for cold shock mRNA (Weber *et al.*, 2001; Mascarenhas *et al.*, 2001). A similar observation was reported for CSPs in *Streptomyces aureofaciens*, which also co-purify with ribosomes (Mikulik *et al.*, 1999). The CSPs may co-purify either due to a direct contact with the ribosome and/or through binding to mRNA, in either case CSPs are positioned at the translation machinery during cold shock. The involvement of a CSP in the repression of translation has been presented. Recently, a cold-induced protein has been identified in *E. coli* that binds to the 30S subunit and 70S ribosomes at the interface between the 30S and 50S subunits (Agafonov *et al.*, 1999; Agafonov *et al.*, 2001; Vila-Sanjurjo *et al.*, 2004). This protein, protein Y, specifically inhibits translation by blocking the binding of aminoacyl-tRNA to the A-site (Agafonov *et al.*, 2001). Interestingly, protein Y was present throughout the cold shock acclimatization phase (first 3 h of cold shock), disappearing at the transition to the cold adaptation phase, suggesting that protein Y is involved in slowing and/or reducing protein synthesis at the translation initiation and elongation step (Agafonov *et al.*, 2001; Vila-Sanjurjo *et al.*, 2004). However, the translation repression during cold shock has also been suggested to be the result of ribosome

trapping by pre-mature mis-folded proteins (Phadtare *et al*, 2000). Indeed, in *E. coli* and *B. subtilis*, peptidyl prolyl isomerases that catalyze the *cis/trans* isomerization of peptide bonds (Graumann *et al*, 1996; Kandor and Goldberg, 1997) are cold induced. These data suggest a role for TF (trigger factor) and PPiB (peptidyl-prolyl cis-trans isomerase) in "maintenance and repair" function by renaturing mis-folded proteins at the ribosome allowing translation to proceed at low temperature and/or promoting refolding of cold-damaged proteins (Phadtare *et al*, 2000).

1.2.3 CSPs role in the adjustment of membrane fluidity

In response to cold shock a decrease in membrane fluidity occurs, where the normal liquid-crystalline phase (fluid-state) undergoes a transition to a gel-phase (non-fluid state) (Yamanaka 1999; Graumman and Marahiel 1999; Weber and Marahiel, 2003; Golovlev, 2004; Phadtare, 2004). The decrease in membrane fluidity results in the decrease or impairment of protein transport and/or membrane associated functions (Pogliano and Beckwith, 1993; Bolhuis *et al*., 1998; Weinberg *et al*., 2005; Han *et al*., 2005). Thus, the adjustment of membrane fluidity, known as homoviscous adaptation, is essential for maintaining the integrity of the membrane in response to cold shock. The reduction in membrane fluidity caused by low temperature is adjusted by extensive desaturation of membrane fatty acids (FA), by FA desaturases (Jones and Inouye 1994; Los and Murata 1998; Jones *et al*., 2002). Increased expression and/or activity of fatty acid desaturase(s) during cold shock has been well documented in bacteria. The increase in desaturation is achieved by acyl-desaturases encoded by *des* genes

(Vigh *et al.*, 1998, Los and Murata 1999). It was first reported that *E. coli* adjusts its fatty acid (FA) composition in response to cold by converting palmitic acid to unsaturated FA cis-vaccenic acid by the β-ketoacyl-synthase II (desaturase) (Garwin and Cronan, 1980). Interestingly, the activity of this enzyme and not its transcription increases significantly in response to cold shock, suggesting that enzyme activity is upregulated by a post-translational modification in response to low temperature (Weber and Marahiel 2003). In contrast, in *B. subtilis*, expression of a cold shock-induced desaturase (Acyl-CoA-desaturase type) is upregulated at the transcription level (Dunkley *et al.*, 1991; Grau and Mendoza, 1993).

1.3 Proposed cold shock sensors

It is now well accepted that bacteria have adopted thermosensing mechanism(s) to adjust to temperature fluctuations. The characteristics of a cold sensor should be similar to any other stress sensor where it would be capable of perceiving a change in the external temperature (cold signal) and then transducing this signal in a rapid and efficient manner allowing for proficient response to cold shock.

In bacteria, a number of cold thermosensors have been proposed, including: DNA conformation, mRNA stability, the state of the ribosomes, the physical state of the cytoplasmic membrane, and proteins acting as temperature sensors. Each proposed cold thermosensor will be discussed in relation to its role and potential position in cold sensing.

1.3.1 DNA conformation

The regulation of chromosomal DNA conformation by maintaining the appropriate degree of supercoiling is important to maintain DNA function and integrity including replication, transcription, and homologous recombination under all conditions including temperature shock (Dorman, 1991). During cold shock, an increase in negative supercoiling was observed in the chromosomal DNA of both *E. coli* and *B. subtilis* (Jones and Inouye 1994, Grau *et al.*, 1994; Mizushima *et al.*, 1997). Negative supercoiling is a function of DNA gyrase, the expression and activity of whose α subunit is cold-induced (Sugino *et al.*, 1977; Jones *et al.*, 1992). Therefore, it has been proposed that the increased negative supercoiling by DNA gyrase in response to cold shock may play an important role in maintaining DNA replication and/or allowing the transcription of cold-induced genes required for response to such shock (Jones and Inouye 1994; Grau *et al.*, 1994; Mizushima *et al.*, 1997). In addition, two other cold inducible *E. coli* genes encode proteins which function to maintain DNA integrity, the well studied RecA, involved in DNA recombination and repair, and H-NS, a nucleoid associated DNA binding protein (Jones *et al.*, 1987). Although the role performed by these proteins in cold shock response remains to be identified, they also potentially function to maintain DNA integrity and/or maintain an appropriate conformation required for the transcription for cold-induced genes. In addition, expression of the β-subunit of the nucleoid-associated histone like protein HU ($2\alpha2\beta$) is cold-induced, and its deactivation resulted in a cold-lethal phenotype, indicating a role for HU in the cold shock response (Wada *et al.*, 1988, Giangrossi

et al., 2002). Transcription of the β-subunit of HU (*hup*) increased immediately after cold shock combined with an increased mRNA stability, followed by the expression of the α-subunit of HU (Giangrossi *et al.*, 2002). Bahloul *et al.* (2001) have suggested that upon cold shock, HU may assist the reloading of DnaB to allow new DNA replication initiation cycle events at *oriC*, thereby inducing new rounds of DNA synthesis preparing the cell for long term survival in response to cold shock.

These results suggest that the selective expression or increased enzymatic activity of proteins required to maintain DNA conformation or integrity are involved in the regulation of gene expression during cold shock. Despite the fact that a change in DNA conformation has been considered as a potential cold thermosensor, it does not have the characteristics of a sensor including: (i) expression of the genes discussed above is regulated by the protein product of other cold-induced genes; CspA for *gyrA* and *hns* and FIS (factor for inversion stimulation) for *hup* (Claret and Rouviere-Yaniv, 1996), indicating that cold shock has a delayed effect on their expression, and thus (ii) eliminating the capability of a rapid response to cold shock. Therefore, the change of DNA conformation is more likely a downstream response to cold shock.

1.3.2 mRNA stability

Increased activity of the cold-induced DNA binding proteins involved in regulation of DNA conformation described above was found to be dependent on increased stability of their mRNA during cold shock (Grau *et al.*, 1994; Giangrossi *et al.*, 2002). This suggests that cold increases mRNA stability and

potentially increased degradation of non cold shock mRNAs may provide or initiate the mechanism which regulates the selective translation of cold shock mRNAs observed during cold shock. Thus, selective and rapid translation of cold shock mRNA would result in, for example CspA synthesis, leading to enhanced *gyrA* and *hns* expression, thereby allowing the adjustment of DNA conformation in response to cold shock. In *E. coli*, *cspA* mRNA has a half-life of >20 min at 15˚C as compared to 12 sec at 37˚C (Fang *et al*, 1997; Mitta *et al*, 1997). This is a common theme reported for most, if not all, cold-induced genes in both Gram-positive and negative bacteria (e.g. Listeria, Lactobacilli, Bacilli, cyanobacteria etc.) (Graumann and Marahiel, 1999; Los and Murata, 1999; Yamanaka, 1999; Chamot and Owttrim, 2000; Wouters *et al.*, 2000). A possible explanation for the increased stability of cold shock mRNA is due to increased thermodynamic stability of secondary structures within the long 5′ UTRs characteristic of the cold shock mRNAs during cold shock. This alteration in mRNA folding could contribute both to the decrease in general translation and in the selective translation of cold shock-mRNA by creating RNase sensitive and insensitive sites within the RNA structures, respectively. The RNA degradosome may be involved in the alteration of mRNA stability upon cold shock. The major components of the degradosome are RNaseE, enolase, RNA helicase (RhlB), and PNPase (Mackie *et al.*, 2001). PNPase is a cold-induced 3′-5′ exoribonuclease (Carpousis, 2002). The *pnp* mutants of both *E. coli* and *Yersinia enterocolitica* are unable to degrade *cspA* mRNA at 37˚C (Neuhaus *et al.*, 2000; Yamanaka and Inouye 2001), leading to the suggestion that PNPase is responsible for the low stability of *cspA*

mRNA at 37°C (Yamanaka and Inouye, 2001). However, at the end of the cold acclimation phase PNPase becomes functional and degrades *cspA* mRNA. Otherwise the massive accumulation of cold shock mRNAs would occupy all ribosomes inhibiting the resumption of normal growth (Yamanaka and Inouye, 2001). Therefore PNPase function is necessary to clear ribosomes by degrading the *csp* mRNA after the cold acclimation phase has been completed. In this scenario, one could propose that the cold shock-induced alteration in mRNA stability may be the mechanism by which selective translation occurs during cold shock (Brandi *et al.*, 1996; Giuliodori *et al.*, 2004). The idea of a cold shock adapted degradosome has recently been proposed. Prud'homme-Généreux *et al.* (2004), show that CsdA co-purifies with RNaseE, and *in vitro* replaces RhlB function. These findings propose a function for the cold-induced RNA helicase, CsdA, in mRNA metabolism during cold shock. Therefore, an increase in the cold-induced mRNA stability would be a potential signal for the cell to initiate the cold shock response. This is an attractive scenario, however, the ribosome has to be alterered to enable it to specifically translate cold shock mRNA.

1.3.3 The state of the ribosome

The primary metabolic effect caused by cold shock is a reduced level of translation as a result of the inability to initiate translation. This reduction is manifested by a decrease in polysomes and an increase in 70S ribosomes and free ribosomal subunits (30S and 50S) (Das and Goldstein, 1968; Friedman *et al.*, 1969a; Broeze *et al.*, 1978; Farewell and Neidhardt, 1998; Yamanaka, 1999;

Bayles *et al.*, 2000). Cells must therefore overcome this block in translation initiation as well as selectively translate mRNA coding for CSPs.

Van Bogelen and Neidhard (1990) were the first to investigate ribosomes as temperature sensors in *E. coli* by utilizing a non-thermal approach inducing heat or cold shock response using antibiotics. They reported that the cold shock inducing antibiotics cause a block at the ribosomal A site. For example, chloramphenicol inhibits peptidyl transferase, and a block at the A site is generated due to its continuous occupation with charged tRNA. These results lead to the suggestion that the ribosome may be the primary sensor for cold shock and thereby a ribosome thermosensing model was proposed (Fig. 1.1) (Van Bogelen and Neidhard, 1990). This model proposes that the concentration of charged tRNA blocking the A site may be the cold signal, as the translational capacity of the ribosome is significantly reduced. Also, the authors were the first to suggest the existence of specialized ribosomes, for translation of either heat shock or cold shock proteins. In support of the proposed model, Brandi *et al.*, 1996, have shown that ribosomes from cold shocked *E. coli* cells translate *cspA* mRNA more efficiently than ribosomes from cells grown at 37°C. This represents the first *in vivo* evidence for the preferential translation of cold shock mRNA. In order for the ribosome to process this selective translation of cold shock mRNA it has to be cold adapted. Indeed, a number of cold-shock induced proteins including translation initiation factors (IF1, IF2, and IF3), CspA, CsdA, and RbfA are ribosome associated, and may contribute to the modulation of the ribosome to become cold adapted. Despite the fact that the ribosome thermosensor model is

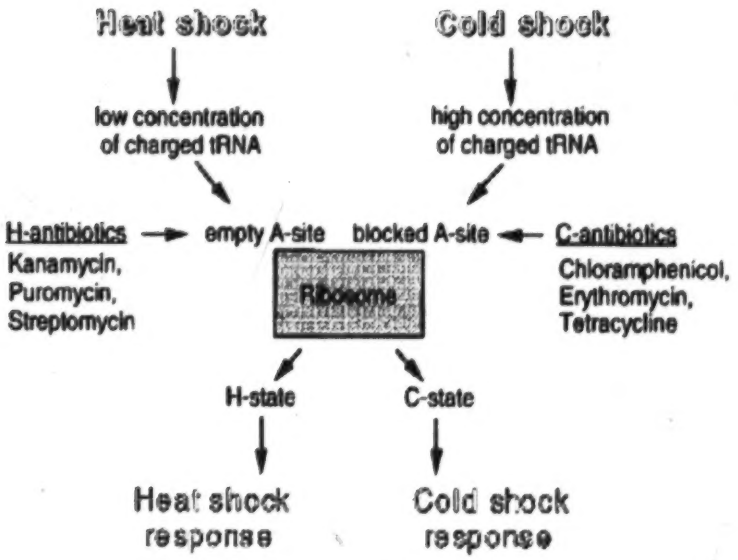


Fig. 1.1. Model of the ribosome as a sensor of temperature in bacteria.

After heat shock, translation proceeds faster than the instantaneous supply of charged tRNA, which may result in an empty A-site, resulting in the heat shock response. In contrast, cold shock causes a reduction in translation due to the high concentration of charged tRNA which in turn block the A-site, thereby eliciting the cold shock response (Van Bogelen and Niedhard, 1990; Graumann and Marahiel, 1996).

still being referred to by many reviewers as a potential cold sensor (Graumann and Marahiel 1996, 1998; Yamanaka 1999; Phadtare *et al*., 2000; Wouters *et al*, 2000; Ramos *et al*., 2001; Golovlev, 2003; Phadtare, 2004), the change in the state of the ribosome and/or factors associated with it, is most probably a result of the cold shock response and does not function as the actual cold sensor. Thus, the altered ribosome may indeed confer a transient preferential translation of mRNA from cold-induced genes, but this is clearly an event which occurs downstream of the thermosensor as the cold shock ribosome is altered by association with proteins which are only present in the cell in response to cold shock. Since the ribosome is not likely to be the prime cold thermosensor, the bacterial cell must either have specialized cold sensor(s) and/or a sensing mechanism(s) that is able to sense and transduce the cold signal.

1.3.4 The physical state of the membrane

Adjustment in membrane fluidity in response to changes in growth temperature is a common response in numerous organisms. This response may be considered a signal for the expression of a set of genes whose products are responsible for reverting the membrane back to its liquid-crystalline phase to maintain fluidity (homoviscous adaptation). As described earlier, at cold temperature the adjustment is accomplished by increasing the level of desaturation in membrane lipids by acyl-desaturases encoded by *des* genes. One such desaturases is the β-ketoacyl-synthase II, whose activity is induced upon cold shock, which may be a result of a post-translational modification in the form of a structural modification similar to M and Tar (Gorwin and Cronan, 1980). In

contrast, cold shock expression of the *B. subtilis* and cyanobacterial *des* genes occurs at the level of transcription (Graumman and Marahiel 1999; Los and Murata, 1999). Des proteins are membrane associated and their possible direct activity in response to a decrease in temperature may identify them as potential cold sensors. In support of this hypothesis, Vigh *et al.* (1993), showed that an artificial decrease in membrane fluidity catalyzed by hydrogenation of fatty acids under isothermal conditions also activated transcription of *desA* in *Synechocystis*. In agreement with these findings, Inaba *et al.* (2003) reported that the cold induction of gene expression in *Synechocystis* was enhanced by the rigidification of membrane lipids that was engineered by double mutation of *desA* and *desD*. These findings suggest that the primary signal in the perception of cold shock is the change in plasma membrane fluidity. This would imply the existence of a membrane associated or integral membrane protein(s), which may be activated by a temperature-induced decrease in membrane fluidity. Activation of this sensor would then be part of or activate a two-component signal transducing cascade, leading to a coordinated response to cold shock response.

1.3.5 Proteins involved in cold signal sensing and signal transduction

The first described thermotactic sensor of cold shock is the aspartate chemoreceptor (Tar) of *E. coli* (Nishiyama *et al.*, 1997). Thermosensing by Tar occurs by reversible methylation of a single residue of the cytoplasmic signaling/attractant domain ($Q_{295}E_{302}Q_{309}E_{491}$) in the presence of an attractant (e.g. aspartate) (Nishiyama *et al.*, 1999). The unmodified form (QEQE) has no thermosensing ability. However, at 37°C (warm), the two glutaminyl residues

(Q_{295} and Q_{309}) are irreversibly deaminated by the CheB deamidase to $E_{295}E_{302}E_{309}E_{491}$ thereby creating a warm sensor. If the thermal signal is cold shock, the $E_{295}E_{302}E_{309}E_{491}$ receptor is methylated to EmEmEmEm by CheR to form a cold sensor (Fig. 1.2) (Nishiyama *et al.*, 1999). Furthermore, Nishiyama *et al.*, (1997) have suggested that the binding of aspartate to Tar may impose a structural constraint on the methylated form that is required for the cold sensor phenotype. This structural constraint has been proposed to involve the formation of a coiled-coil motif between two cytoplasmic signaling domains, MH1 and MH2 (Surette and Stock 1996; Nishiyama *et al.*, 1997). The coiled-coil motif role in cold thermosensing has also been described for the membrane associated M proteins of *Streptococcus* group A, where some classes of M proteins appear as coiled-coil dimers at low temperature, but as monomers at 37°C (Cedervall *et al*, 1997). Therefore, it has been suggested that M proteins can sense temperature via the formation of a coiled-coil motif as a result of M protein dimer (Cedervall *et al.*, 1997). However, how alterations in Tar and M protein conformation are transduced into the cold shock response must involve upstream events.

These events most likely involve a two-component signal transduction pathway. Indeed, CheB is activated after receiving a phosphate group from the histidine kinase CheA. Binding of a repellant or an attractant to CheA (chemoreceptor) activates or inactivates its autophosphorylation (Nishiyama *et al.*, 1999). Thus, the phosphorylation state of CheA may be the signal required for temperature sensing that is then transduced to response regulators (e.g. CheB).

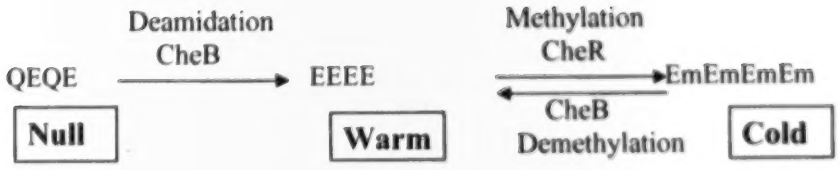


Fig. 1.2. Effects of covalent modification on thermosensing of Tar (Nishiyama et al. 1999). Tar methylation and demethylation are responsible for cold and warm adaptation respectively. CheR catalyses the transfer of a methyl group from S-adenosylmethionine to a glutamyl side-chain, and CheB hydrolyses the methyl-ester bond of a methylated glutamyl residue. The latter enzyme is also a deamidase that converts glutaminyl residues at methylation sites to glutamyl residues.

Although this may position CheA as a potential thermosensor, its phosphorylation status is dependent on the binding of a ligand (e.g. aspartate) and not upon perceiving a thermal signal *per se*, thereby, its description as a chemoreceptor may be a more accurate description of its function.

A two-component signal transduction system has been implicated as a cold thermosensor in *B. subtilis* (Aguilar *et al.*, 2001). This proposed sensor is composed of a sensor kinase, DesK, and a response regulator DesR, responsible for the cold induction of the *des* gene expression coding for a Δ5-lipid desaturase (Aguilar, 2001). Similar to the CheA/B system described above, recently it has been reported that the level of DesR phosphorylation regulates the expression of *des* (Cybulski *et al.*, 2004). These findings provide the first evidence for a transcription factor functioning as a phosphorylation-activated switch regulating expression of a cold shock-induced gene (Cybulski *et al.*, 2004). A major problem with these observations is that DesR only regulates the expression of a single gene, *des*, which suggests the existence of other regulator(s) for the expression of other cold-induced genes.

A similar signaling system was identified in the cyanobacterium *Synechocystis*, where disruption of all 54 of the histidine kinase genes revealed that Hik33 (sensor kinase) and Hik19 (response regulator) were necessary for low temperature induction of *desB* expression (Suzuki *et al.*, 2000 and 2001). However, despite the fact that inactivation of Hik33 and Hik19 reduced the cold shock induction of *desB*, a minimal effect on expression of two other cold-induced genes, *desA* and *crh* (cold-induced RNA helicase), was observed. This

again suggests the existence of a separate, or even multiple 2 component signal transduction pathways, regulating CSP gene expression during cold shock in this organism. On the basis of these results, Suzuki *et al.* (2000 and 2001) have presented a model suggesting that Hik33 autophosphorylation is activated by the reduction in membrane fluidity (Fig.1.3). For Hik33 to be an actual cold thermosensor it would have to be membrane associated and/or interact with a temperature sensing protein(s) in the membrane, allowing its phosphorylation state to be regulated by the change in membrane fluidity.

It is possible that these sensor kinases may not be activated by a change in membrane fluidity or reduction in temperature and therefore may also be tagets downstream of the actual cold sensors. A more elaborate cold sensing system has been recently described in mammalian systems. The cold sensor in mammalian cells is an integral membrane protein that functions as an ion channel controlling calcium entry. This cold sensor belongs to the transient receptor potential (TRP) channel protein family (Reid and Flonta, 2001; Peier *et al.*, 2002). A member of this family, TRPM8, is specifically expressed in a subset of pain and temperature sensing neurons in the skin. TRPM8 was found to be activated by cold temperature and cooling agents, stimulating calcium influx into cells resulting in increased levels of intracellular calcium (Reid and Flonta, 2001; Peier *et al.*,

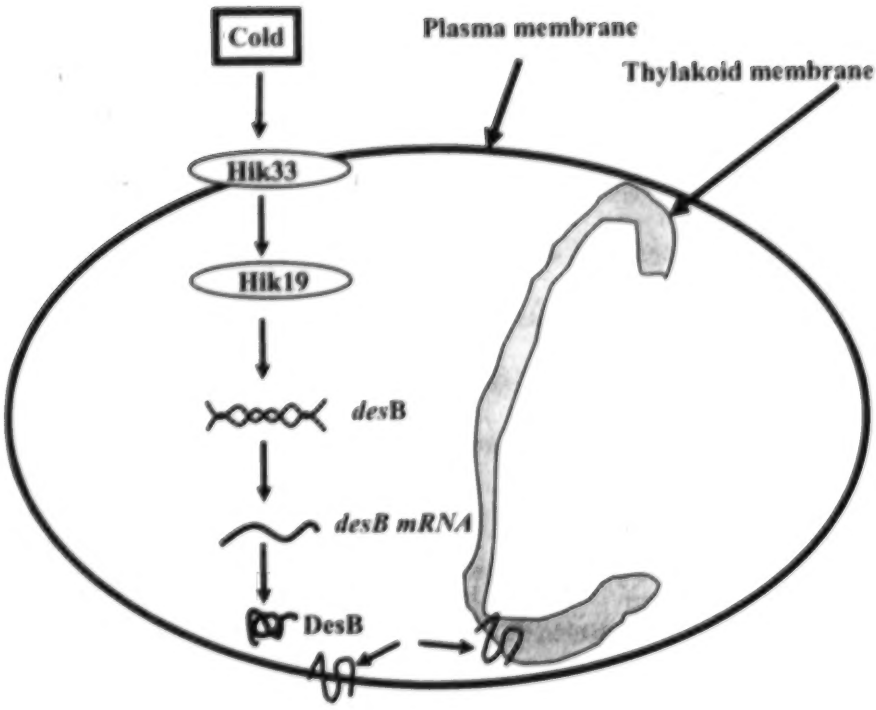


Fig. 1.3. A hypothetical scheme of the signal transduction pathway for low-temperature in *Synechocystis* (Adapted from Suzuki *et al.*, 2000).

When the temperature is decreased or the fatty acids are more saturated, the histidine residue in the histidine kinase domain of Hik33 may be phosphorylated. A phosphate group is then transferred to Hik19, which regulates the expression of the *desB*.

2002). Similar to mammalian cells, plants also sense cold through temperature induced changes in plasma membrane fluidity communicated through altered fluxes in Ca^{2+} levels. This leads to activation of calcium dependent protein kinases (serine/threonine and tyrosine) such as the mitogen-activated protein kinase (MAPK) family, allowing downstream signal transduction (Hirt, 2000; Sangwan *et al.*, 2002). Although a similar mechanism has yet to be described in bacteria, finding a bacterial homologue(s) of TRPM8 or MAPK would initiate molecular studies to solve the problem of how prokaryotes perceive cold. In support of this proposal, Torrecilla and co-workers (2000) reported that cytosolic Ca^{2+} is mobilized in *Anabaena* in response to a temperature change, where the Ca^{2+} source for cold shock signaling is mostly extracellular.

These findings strengthen the assumption that a decrease in the membrane fluidity may be the prime cold sensor, where Ca^{2+} influx may activate a phosphorylation cascade regulating the expression of cold shock-induced genes and resulting in cold shock adaptation.

1.4 Cyanobacteria as a model system for the study of the cold shock response

Cyanobacteria represent one of the most ancient forms of life and comprise a diverse class of Gram negative bacteria characterized as being obligate photoautotrophs which perform oxygen evolving photosynthesis analogous to chloroplasts (Stanier and Cohen-Bazire, 1977). They are widely distributed and thriving in a diverse range of temperature habitats, ranging from Antarctica (Psenner and Sattler, 1998) to hot springs (Ward *et al.*, 1998). In addition, cyanobacteria are capable of cellular differentiation. For example, vegetative cell

terminal differentiation to heterocysts (cells specialized for nitrogen fixation) in nitrogen limiting environments (Fay, 1983), or akinetes (equivalent to bacterial spores) or hormogonia (whole filaments specialized for motility) (Schopf, 1996; Psenner and Sattler, 1998; Ward *et al.*, 1998).

In cyanobacteria there are three structurally and functionally distinct membranes; the outer membrane, the plasma membrane and the thylakoid membrane (Fig. 1.4). Although the photosystems are restricted to the thylakoid membrane, contact points have been noted between the plasma membrane and the thylakoid membrane. It is well documented that the photosynthetic and respiratory electron transport chains share electron carriers in the cyanobacterial thylakoid membrane (Peschek *et al.*, 1989). The plasma membrane can also serve as a photosynthetic membrane, as in the cyanobacterium *Gloeobacter violaceus* which lacks a thylakoid membrane (Guglielmi *et al.*, 1981). The general structural and functional features of the cyanobacterial plasma and thylakoid membranes are similar to those of higher-plant chloroplast thylakoids (Los and Murata, 1999). This observation supports the hypothesis that chloroplasts originated from a symbiotic relationship between cyanobacteria and nucleated cells (Schopf, 1996). Therefore, cyanobacteria provide a powerful model system for the study of stress responses and acclimation mechanisms in both bacteria and plants.

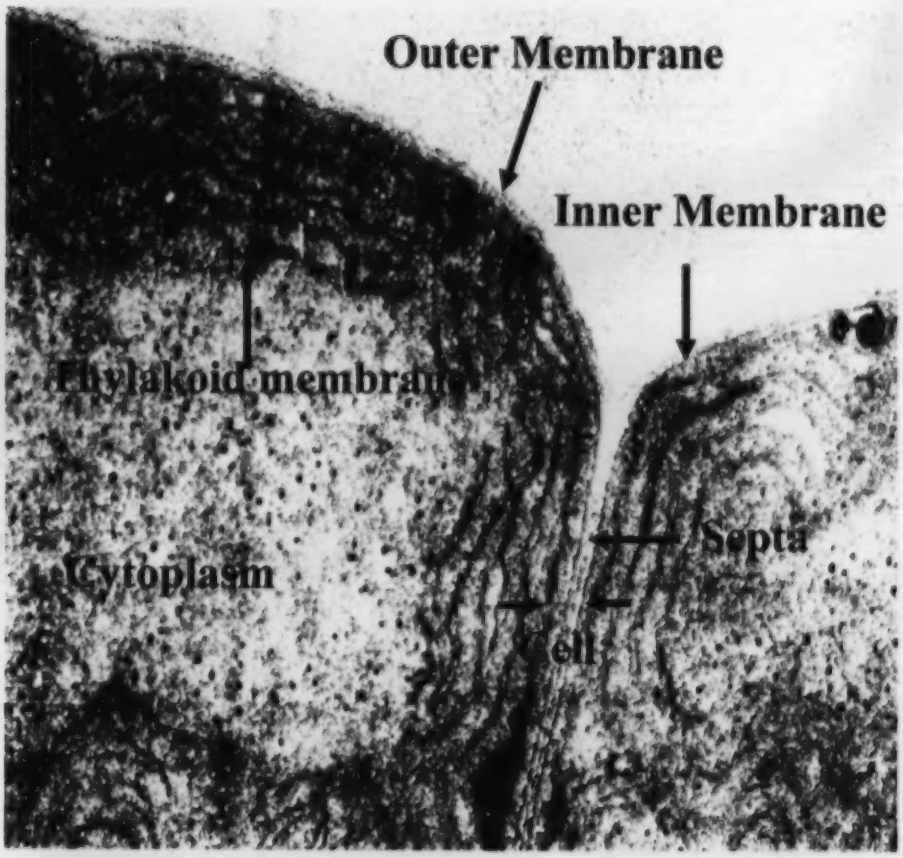


Fig 1.4. Transmission electron microscopy image of the filamentous cyanobacterium *Anabaena* sp. strain PCC 7120 (Bassam and Owttrim, unpublished work).

The arrows indicate the subcellular organization of the outer, plasma, and thylakoid membranes, the septum separating two cells, and the cytoplasm. Bar=500 nm.

1.4.1 *Anabaena* sp. strain PCC 7120

The cyanobacterium *Anabaena* sp. strain PCC 7120 is a filamentous obligate photoautotroph (Fig. 1.4) and was the primary model organism used in this study. The complete 6.4 megabase genome of *Anabaena* was elucidated by Kaneko *et al.* (2001) and is available (http://www.kazusa.or.jp/cyano/Anabaena). Availability of the complete genome, together with well established gene transfer protocols, mutagenesis, and reporter gene expression systems for *Anabaena* make it a model organism to study stress responses, oxygenic photosynthesis, nitrogen fixation, and cellular differentiation.

One of these stresses, cold shock, is the subject of this thesis. An example of a cold shock regulated gene from *Anabaena* is the RNA helicase, CrhC (Chamot *et al.*, 1999; Chamot and Owttrim, 2000). This RNA helicase has been reported to be part of a protein complex and proposed to be associated with the ribosome, thus positioning CrhC at the protein synthesis centre (Yu and Owttrim, 2000). The subcellular localization of CrhC is suggestive of its role in cold shock adaptation and identification of proteins that interact with it will help to clarify its role.

1.4.2 Cold-shock in cyanobacteria

Although the acclimation of cyanobacteria to cold shock also involves induction of the expression of a specific set of cold shock-induced genes, the cold shock proteomics of cyanobacteria is not as well studied as in *E. coli* and *B. subtilis*. The mRNA of cold shock-induced genes share the common feature of long 5`UTRs which is characteristic of mRNAs of previously identified cold

shock genes. Cold shock-induced genes of caynobacteria are listed in Table 1.2. Similar to other bacteria, genes whose protein products are involved in maintaining protein synthesis and membrane fluidity predominate.

1.4.3 Cyanobacterial cold-induced genes involved in translation

Although there are no CspA homologs in cyanobacteria, a number of CSPs proposed to be involved in alleviating the block in translation initiation have been identified. Among the first identified cold-induced proteins in cyanobacteria are the RNA-binding proteins (Rbps). Rbps are known to be involved in various aspects of RNA metabolism, such as stability, processing, modification, and translation and have therefore been proposed to replace the CspA family in cyanobacteria (Kenan *et al.*, 1991; Nagai *et al.*, 1995). Sato, (1995) demonstrated that expression of 4 of the 8 *rbp* genes in *Anabaena variabilis* strain M3 (*rbpA1*, *rbpA2*, *rbpA3*, and *rbpC*) are regulated by cold. Rbps also bind RNA specifically, similar to the members of CspA family (Sato, 1995). It has been suggested that Rbps may function similarly to the CSPs in the destabilization of secondary structures in cold shock mRNA facilitating translation initiation (Sato, 1995). A number of genes encoding ribosome-associated proteins are cold regulated in cyanobacteria as was the case in *E. coli* and *B. subtilis*. The ribosomal protein S21 of the 30S subunit is induced by cold and accumulates transiently in ribosomes at low temperature in *Anabaena variabilis* strain M3 and *Synechocystis* (Sato, 1994; Sato *et al.*, 1997). These findings suggest a role for S21 and Rbps in adjusting the ribosome in response to cold shock (Sato *et al.*, 1997; Los and Murata, 1999).

Table 1.2. Genes known to be induced at both transcript and protein level by cold shock in cyanobacteria and their proposed functions in cold shock (List is adapted from Los and Murata 1999; Chamot *et al.*, 1999[a]; Suzuki *et al.*, 2001[b]; Hossain and Nakamoto, 2002 and 2003[c])

Gene product	Organism	Function of protein product
Desaturase family		Adjustment of membrane fluidity
desA	*Synechocystis* sp. PCC 6803 *Synechocystis* sp. PCC 6714 *Synechococcus* sp. PCC 7002 *Spirulina platensis*	
desB	*Synechocystis* sp. PCC 6803 *Synechococcus* sp. PCC 7002	
desC	*Synechococcus* sp. PCC 7942 *Synechococcus* sp. PCC 7002	
desD	*Synechocystis* sp. PCC 6803 *Spirulina platensis*	
RNA binding family		RNA binding/chaperone
rbpA1	*Anabaena variabilis* M3	
rbpA2	*Anabaena variablis* M3	
rbpA3	*Anabaena variabilis* M3	
rbpC	*Anabaena variabilis* M3	
RNA helicases		RNA unwinding
crhB[a]	*Anabaena* sp. PCC 7120	
crhC[a]	*Anabaena* sp. PCC 7120	
crhR[b]	*Synechocystis* sp. PCC 6803	
Clp family		
clpB	*Synechococcus* sp. PCC 7942	Molecular chaperone
clpP1	*Synechococcus* sp. PCC 7942	Protease
clpX	*Synechococcus* sp. PCC 7942	Unknown
Others		
rpsU	*Anabaena* variabilis M3	30S ribosomal subunit (S21)
lit2	*Anabaena* variabilis M3	Unknown
hptG[c]	*Synechococcus* sp. PCC 7942	Unknown

Three RNA helicases in cyanobacteria have been found to be cold-induced (Chamot, *et al.*, 1999; Kujat and Owttrim, 2000). Although ribosome association has not been reported, expression of the redox regulated cyanobacterial RNA helicase (CrhR) from *Synechocystis* is significantly increased in response to cold shock (Kujat and Owttrim, 2000). Furthermore, two cold shock-induced genes encoding RNA helicases, CrhB and CrhC, were also identified in *Anabaena* sp. strain PCC 7120 (Chamot *et al.*, 1999). The *crhB* gene is expressed under a variety of stress conditions (salt shock, nitrogen limitation) including cold shock and may represent a homologue of CrhR, while *crhC* expression occurs exclusively in response to cold shock (Chamot *et al.*, 1999; Chamot and Owttrim, 2000). Moreover, CrhC is a component of a multi-subunit complex during cold shock, although the identity of the associating proteins is not known (Yu and Owttrim, 2000). The role of CrhC in cold acclimation is proposed to be in the active unwinding of 5′ UTR secondary structures of cold shock-induced mRNAs, allowing cells to remove the cold induced blockage of translation initiation (Chamot *et al.*, 1999; Yu and Owttrim 2000).

A potential cause of translation repression during cold shock is the trapping of the ribosome by premature mis-folding of proteins rendering ribosomes unavailable for new rounds of protein synthesis (Graumann *et al*, 1996; Kandor and Goldberg, 1997). The degradation of such proteins appears to be a necessity to alleviate translation repression, thus allowing the recycling of the ribosome. A new family of bacterial molecular chaperones that includes proteases is known as the caseinolytic proteases (Clps), have been identified in

cyanobacteria. The growth of the *clpI* null mutant of *Synechococcus* is severely inhibited when the culture is shifted from 37°C to 25°C (Porankiewicz and Clarke, 1997). Also, ClpB expression in *Synechocystis* is induced in response to cold shock (Porankiewicz and Clarke, 1997; Celerin *et al.*, 1998). Mutagenesis of the *clpB* gene accelerated the inhibition of photosystem II activity at low temperature, leading to a reduction in growth rate (Porankiewicz and Clarke, 1997; Schelin *et al.*, 2002). Porankiewicz and Clarke (1997) suggested that ClpB might function in the renaturing of mis-folded proteins that are trapping the ribosome. This would clear the ribosomes of mis-folded proteins allowing subunit recycling and thus aiding in alleviating the repression in translation during cold shock (Los and Murata, 1999).

1.4.4 Cold shock effect on membrane fluidity in cyanobacteria

The cold shock response in cyanobacteria has been more extensively investigated in respect to changes in membrane fluidity. Four desaturase genes have been identified in *Synechocystis*, *desA*, *desB*, *desC*, and *desD* (Los *et al.*, 1997). Expression of *desA*, *B*, and *D* increase at least 10-fold in response to cold shock, while *desC* expression levels remain unchanged (Los *et al.*, 1997). All four desaturases are associated with both the thylakoid and the cytoplasmic membrane, suggesting that fatty acid desaturation takes place in both membranes (Mustardy *et al.*, 1996). Although desaturase gene expression has been mainly studied in *Synechocystis*, there are several reports of cold-induced desaturase genes in other cyanobacteria. For example *desC* in *Synechococcus* is also cold-induced (Ishizaki-Nishizawa *et al.*, 1996; Sakamoto *et al.*, 1997). These observations suggest that

desaturases in cyanobacteria and their differential expression in response to reduced temperature play a central role in adjusting membrane fluidity during cold shock (Los and Murata, 1999; Inaba *et al.*, 2003).

In summary, formation of a cold-adapted ribosome and adjustment of membrane fluidity are the two major requirements for cellular adaptation to cold shock. Thus, it is important to investigate the mechanism by which these alterations are accomplished in order to understand the cold shock response.

1.5 Thesis objective

In this thesis, I propose an expanded model for the cold shock response and adaptation in cyanobacteria. As described earlier, cold shock mainly affects protein synthesis and membrane fluidity. Cold shock research is therefore focused on understanding how the cell overcomes these physiological limitations. The research presented in this thesis is composed of two parts that are designed to uncover components of the cold shock response and adaptation pathway and thus advance our understanding of cellular response to cold shock in cyanobacteria. The first part involves investigation of a potential role(s) for CrhC in cold shock response and adaptation in cyanobacteria by subecellular localization of CrhC and identification of proteins which interact with CrhC. Previous work in our lab has identified and biochemically characterized a cold-induced RNA helicase, CrhC, (Chamot, *et al.*, 1999). Furthermore, immunoprecipitation and Far-Western analysis showed that CrhC is present in a multi-subunit complex, whose members remain to be identified (Yu and Owttrim, 2000). Cellular localization and identification of proteins interacting with CrhC will suggest the cellular

function(s) of this cold-induced RNA helicase in the cold shock response and adaptation in cyanobacteria. The second part of this thesis involves identification of downstream targets of the cold shock signal transduction pathway. Identification of these target(s) allows a more precise description of the mechanism by which cold shock leads to a reduction of general protein synthesis as well as to new targets of a cold shock signal transduction pathway. The findings presented in this thesis combined with current findings from the cold shock literature enable me to propose an advanced model for cold shock response and adaptation in cyanobacteria which should be applicable to bacteria in general.

Chapter 2

Materials and Methods

2.1 Localization of CrhC in cold shocked *Anabaena* sp. strain PCC 7120

2.1.1 Bacterial strains and culture conditions

The bacteria used in this study, their relevant genotypes, and their sources are listed in Table 2.1 and 2.2. *Anabaena* sp. strain PCC 7120, obtained from the University of Toronto culture collection (UTCC 387), was grown photoautotrophically at 30°C in BG-11 liquid medium (Allen, 1968) with constant illumination (cool white fluorescent light, 150 microeinsteins/m²/sec) and referred to as warm grown cells. Liquid cultures were aerated both by shaking at 120 rpm, and continuous bubbling with sterile filtered (0.45 μm filter) (Millipore, Cambridge, ON, Canada) humidified air, and grown to mid-log phase (A_{750nm}= 0.6). Cold shocked cells were obtained by transferring mid-log phase liquid cultures to 20°C for the indicated times. Cells were harvested by centrifugation at the stated growth temperature, washed with 20 mM Tris-HCl pH 8.0, 10 mM NaCl, 1 mM EDTA at the appropriate temperature, frozen in liquid nitrogen, and stored at -80°C. *E. coli* DH5α (Invitrogen, Burlington, ON, Canada) and JM109 (Stratagene, La Jolla, CA, USA) cells were grown in LB medium (Luria broth medium) (Difco, BD Diagnostics, NJ, USA). When required, ampicillin was included at a final concentration of 100 μg/ml.

2.1.2 Protein extraction and immunoblot analysis

Frozen cells were thawed on ice, resuspended in ice cold PBS-I (4.3 mM $Na_2HPO_4.7H_2O$, 1.4 mM KH_2PO_4, 137 mM NaCl, and 2.7 mM KCl pH 7.4) containing complete mini protease inhibitor cocktail (Roche, Mississauga, Ontario, Canada), and mechanically lysed by vortexing at top speed for 30 seconds 5 times followed by 1 minute in an ice bath in the presence of an equal volume of glass beads (0.2-0.3 mm)

Table 2.1. Cyanobacterial strains used in this study

Strain	Relevant genotype	Reference/Source	Use
Anabaena sp. strain PCC* 7120	Wild type	University of Toronto culture collection (UTCC)	Study subject
Synechocystis sp. strain PCC 6803	Wild type	University of Toronto culture collection (UTCC)	Control
Synechococcus sp. strain PCC 7942	Wild type	University of Toronto culture collection (UTCC)	Control

* Pasteur Culture collection

Table 2.2. *Escherichia coli* strains utilized for the overexpression of CrhC and CrhR

Strain and relevant genotype	Plasmid	Reference/Source	Use
Escherichia coli JM109 *recA supE44 endA1 hsdR17 gyrA96 relA1 thi Δ(lac-proAB) F' [traD36 proAB⁺ lacᵠ lacZΔM15*	pRSET29 [Amp^R *crhC*]	Yu and Owttrim 2000	His_6-CrhC overexpression
	pRSET:*crhR* [Amp^R *crhR*]	Kujat and Owttrim 2000	His_6-CrhR overexpression

(Impandex, Clifton, NJ, USA). Pellet fractions were resuspended in PBS-I in a volume equal to that of the supernatant. CrhC was solublized from the pellet fraction by incubation in denaturing buffer (PBS-I, 1% SDS w/v) for 10 min on ice. Where indicated, pellet fractions were resuspended in PBS-I and incubated with either DNase I (3 U/50 µg protein) (Roche, Mississauga, Ontario, Canada) or RNase A (5 U/50 µg protein) (Roche, Mississauga, Ontario, Canada) at 37°C for 20 min. After treatment, the mixtures were centrifuged at 11 000 x *g* at 4°C for 20 min to produce supernatant and pellet fractions and analyzed by immunoblotting. Protein concentration was quantified using the Bradford protein assay (Bio-Rad, Mississauga, Ontario, Canada) with bovine serum albumin (BSA) (Sigma, St Louis, MO, USA) as the standard. Polypeptides were separated by 10% SDS-PAGE, and transferred to nitrocellulose membranes (Hybond ECL, AmershamPharmaciaBiotech, Piscataway, NJ, USA), under constant electric current (60 milliampers for 45 minutes) using a semidry apparatus (Tyler Research, Edmonton, AB, Canada). All components including Whatman 3 MM paper and 0.45 micron nitrocellulose filters (AmershamPharmaciaBiotech, Piscataway, NJ, USA) were soaked in 1 X transfer buffer (25 mM Tris/HCl: 150 mM glycine pH 8.3; 20% (v/v) methanol) for 30 min before transfer. After electroblotting, membranes were blocked with 5% non-fat powdered milk in Tris-buffered saline (TBS, pH 8.0) and incubated with the indicated polyclonal primary antibodies (against CrhC, CpxA, or Rps1) at 1:5 000 dilution for 1 h. Blots were washed for 5 min each consecutively with TBS, TBST (TBS with 0.1% Tween 20) ((Sigma, St Louis, MO, USA), and TBS, incubated with secondary antibody linked to

horseradish peroxidase (Sigma, St Louis, MO, USA) at 1:20 000 dilution for 20 min, washed as described above, and visualized by chemiluminescence (ECL, AmershamPharmaciaBiotech, Piscataway, NJ, USA). Immunoblots were stripped by incubation in stripping buffer (62.5 mM Tris-HCl pH 6.8, 2 % SDS, 100 mM β-mercaptoethanol) at 50°C for 30 min followed by extensive washing in 1 X TBS. Anti-CrhC antiserum was generated as described (Yu and Owttrim, 2000). Antiserum raised against the *E. coli* ribosomal protein S1 (Rps1) and the histidine kinase CpxA were generously provided by Dr. P. Baumann (University of California) and Dr. T. Raivio (University of Alberta), respectively.

2.1.3 Membrane association and orientation

Spheroplasts were prepared as described (Owttrim and Colman, 1986) with slight modifications. Spheroplasts were generated by incubation of *Anabaena* cells in PPS (30 mM potassium phosphate pH 7.5, 0.55 M sorbitol) with 0.1% (w/v) lysozyme at 37°C for 2 h and washed extensively with PPS. Spheroplasts were lysed mechanically by vortexing in an equal volume of glass beads, centrifuged at 1 000 x *g* to remove unbroken spheroplasts, and the supernatant centrifuged at 14 000 x *g* for 30 min to produce a crude membrane-containing pellet. Aliquots of this fraction (20 µg protein) were treated with the indicated denaturants by incubation on ice for 30 min, except for SDS which was performed at room temperature. The samples were centrifuged at 14 000 x *g* for 30 min at 4°C and the resulting supernatant and pellet fractions (5 µg protein) analyzed by immunoblotting. CrhC orientation in the plasma membrane was determined by incubating spheroplasts (300 µg protein) in PPS containing trypsin

(30 µg/ml) (Sigma, St. Louis, MO, USA) at room temperature. Trypsin digestion was terminated by addition of soybean trypsin inhibitor (40 µg/ml) ((Sigma, St Louis, MO, USA), EDTA (5 mM), and PMSF (10 mM) (Sigma, St Louis, MO, USA)). The trypsin treated spheroplasts were washed in PPS, mechanically lysed, and the total lysates (5 µg protein) analyzed by immunoblotting.

2.1.4 Fractionation of *Anabaena* cell membranes

Membrane fractionation was performed by flotation centrifugation on a discontinuous sucrose density gradient as previously described (Murata and Omata, 1988). Spheroplasts were lysed by a single passage through a French press (40 MPa) and the homogenate centrifuged at 5 000 *g*. The clarified homogenate was brought to 50% sucrose by addition of 0.8 volumes of a 90% (w/v) sucrose solution. A 17-ml aliquot of this suspension was placed in the bottom of a 35 ml centrifuge tube and overlaid with 8, 3, and 7 ml of sucrose solutions of 39%, 30% and 10%, respectively. Membranes were separated by centrifugation in a swinging-bucket rotor at 130 000 x *g* for 16 h at 4°C. Plasma membranes form a yellow band in the 30% layer, a mixture of plasma and thylakoid membranes forms at the 30 to 39% interface, while thylakoid membranes form a green band at the 39-50% interface. Cell wall material collects as a pellet.

2.1.5 Protein analysis programs

Analysis for potential protein transmembrane domains was performed using PepTool 1.1 (BioTools) and DAS (Cserzo *et al.*, 1997). Predicted protein localization (cytoplasmic, transmembrane, lipoprotein) was analyzed using PSORT. PSORT and DAS were obtained from the ExPASy-Tools web site

(ca.expasy.org/tools). CrhC was also analyzed as a potential lipoprotein using the program DOLOP obtained from www.mrc lmb.cam.ac.uk/genomes/dolop/analysis.htm.

2.1.6 Phospholipase treatment

Purified plasma membranes from cold shocked *Anabaena* sp strain 7120 were treated with phospholipase C III, Phospholipase CIX, phospholipase C PI specific, and phospholipase D (Sigma, St. Louis, MO, USA). The membranes were suspended in digestion buffer (0.13 M NaCl, 20 mM sodium phosphate pH 7.4) to a concentration of 1 unit phospholipase/100 µg protein. Samples were incubated at 37°C for 30 min. Then centrifuged, the resulting pellet and supernatant were analyzed by 10% SDS-PAGE and Western blotting.

2.1.7 Identification of the CRD epitope on the phospholipase C released CrhC

Phosphlipase C PI specific treated membranes were resolved by SDS-PAGE, and then analyzed by Western blotting as described by Hooper *et al.* (1991). Briefly, the blots were blocked with 0.5 % (v/v) Tween 20 for 1 hour at room temperature. The blots were subsequently incubated with a 1:3 000 dilution of anti-CRD antibodies as the primary antibody (Gift from Dr. Hooper, university of Leeds, UK), following steps for washing and developing of the blots were as described in section 2.1.2. Phospholipase C PI specific solubilized pig membrane dipeptidase (MDP 2 µg/10 µl) was used as a control (Gift from Dr. Hooper, University of Leeds, UK,).

2.1.8 Mild acid treatments of phospholipase C treated CrhC

Aliquots of supernatant resulting from PI specific phospholipase C treatment were subjected to deamination by 0.25 M HNO_2 (0.25 M sodium acetate/0.25 M $NaNO_2$, pH 5) for 3 h at room temperature, and with 1 M HCl for 30 min at room temperature (Hooper, 1992). The samples were neutralized with NaOH, and immunoblotted as described above with anti-CRD antibodies, and with anti-CrhC antibodies as the primary antibodies. MDP was used as a control.

2.1.9 Immunoelectron microscopy (IEM)

IEM was performed as described (Mustardy *et al.*, 1996) with slight modification. Cells were pelleted by centrifugation at 8 500 x *g* for 10 min and fixed in a mixture of freshly prepared 4 % formaldehyde (v/v) with 0.8 % glutaraldehyde (v/v) in 0.1 M sodium phosphate buffer (pH 7.2) for 30 to 60 min. Samples were washed in 0.1 M phosphate buffer saline (pH 7.2) and dehydrated by incubation in an ascending ethanol series (20 to 90 %). Infiltration and embedding in LR white resin (London Resin Company Ltd., Reading Berkshire, England) was performed by incubation in LR White-ethanol for 4 h, 2 to 3 changes of pure LR white over 2 h, and pure LR white overnight at 50°C. Serial ultrathin sections were mounted on copper grids and incubated in PBG blocking solution (10 mM sodium phosphate pH 7.2, 600 mM NaCl, 0.5% BSA and 0.5 % fish gelatin; Sigma) for 10 min. Grids were incubated in the indicated primary antibody (1:100 dilution) in PGB at room temperature for 1 h. Grids were extensively washed with PBS and incubated at room temperature for 1 h with goat anti-rabbit IgG coupled to 10-nm colloidal gold particles (Sigma, St Louis, MO,

USA)) diluted 1:20 in PBG as the secondary antibody. Sections were washed extensively in PBST (0.1 % Tween 20 in PBS) and stained with uranyl acetate (4 %). Sections were examined with a Hitachi transmission electron microscope (M-7 000, Rexdale, ON, Canada) at 70 kV.

2.2 Identifying proteins interacting with CrhC during cold shock

2.2.1 Cyanobacterial Protein extraction

Spheroplasts were generated from frozen cells as described in section 2.1.1. and mechanically lysed as described in section 2.1.2. Lysates were clarified at 1 000 x *g*, followed by centrifugation at 14 000 x *g* for 20 min at 4°C to produce pellet and supernatant fractions. The supernatant was removed and kept on ice (supernatant I). In order to solubilize peripheral membrane proteins, the pellet fraction was resuspended in 0.1 M carbonate buffer pH 11 containing complete mini protease inhibitor cocktail (Roche, Mississauga, Ontario, Canada), incubated on ice for 30 min, centrifuged at 4°C and the supernatant obtained (supernatant II). Supernatant fractions I and II were mixed together and subjected to overnight dialysis (Spectra, 10 kDa cut off) (Rancho Dominguez, CA, USA) in several changes of PBS-I containing complete mini protease inhibitor cocktail (Roche, Mississauga, Ontario, Canada). The protein preparation obtained was used directly in pull-down and/or immunoprecipitation experiments.

2.2.2 Immunoprecipitation

2.2.2.i Coupling anti-CrhC antibody to protein A immobilized on sepharose beads

Coupling anti-CrhC to protein A was performed as described by Harlow and Lane (1988). Briefly, 100 mg of lyophilized protein A immobilized on Sepharose CL-4B (Sigma) were swollen for 30 min at room temperature in 2 ml of immunoprecipitation coupling buffer (100 mM $NaHCO_3$, 50mM NaCl, 1mM PMSF, pH 8.3). Anti-CrhC antibodies were mixed with swollen protein A beads at a final dilution of 1:50 and the mixture incubated at room temperature for 60 min with continuous end-over-end gentle rotation. The beads were washed twice with 10 volumes of borate buffer (0.2 M sodium borate, pH 9) followed by centrifugation at 1 000 x *g* for 5 min and were resuspended in 10 volumes of borate buffer. For crosslinking, anti CrhC antibodies with protein A solid dimethyl pimelimidate dihydrochloride (Sigma, St Louis, MO, USA) were added to a final concentration of 20 mM, and the mixture incubated for 30 min at room temperature. The reaction was stopped by washing the beads twice in 0.2 M ethanolamine pH 8 (Sigma, St Louis, MO, USA), and then incubating for 2 h at room temperature in 0.2 M ethanolamine with gentle mixing. After the incubation period, the 0.2 M ethanolamine was discarded after pelleting the beads by centrifugation for 1 minute at 11 000 x *g*. Then, the pelleted beads were resuspended in PBS with 1 mM PMSF (Sigma, St Louis, MO, USA) to be used for immunoprecipitation experiments.

2.2.2.ii Immunoprecipitation of CrhC from *Anabaena* cell lysates

Spheroplasts generated from *Anabaena* cells cold shocked at 20°C for 6 h were mechanically lysed as described in section 2.1.2. Lysates were clarified at 1 000 x *g*, followed by centrifugation at 14 000 x *g* for 20 min at 4°C to produce pellet and supernatant fractions. The supernatant fraction was removed and the pellet fraction was washed 3 X in PBS-I buffer. The pellet fraction was resuspended in 100 µl of 1% sarcosyl and kept on ice for 30 min, centrifuged at 14 000 x *g* for 30 min at 4°C and the resulting supernatant diluted 10-fold with immunoprecipitation coupling buffer (100 mM $NaHCO_3$, 50 mM NaCl, 1 mM PMSF, pH 8.3). The diluted protein preparation was mixed with Protein A-anti-CrhC covalently coupled beads (100 µl bed volume) (2.2.2.i). The mixture was incubated at room temperature for 60 min with continuous end-over-end gentle rotation. The sepharose beads were pelleted by centrifugation at 11 000 x *g* for 5 sec, and the supernatant carefully removed. The beads were washed for 5 min consecutively with 2 X 1 ml PBS, 1 X 1 ml PBS with 0.1% Tween 20, and 2 X 1 ml PBS. SDS-PAGE sample buffer (50 µl) was added to the beads and the samples heated at 100°C for 5 min. After 5 sec centrifugation at 11 000 x *g*, the polypeptides were separated by SDS-PAGE gel, and co-immunoprecipitated polypeptides were visualized after Coomassie blue staining.

2.2.3 His_6-CrhC fusion protein expression in *E. coli*

Cloning of *crhC* or *crhR* into pRSETA, a T7 RNA polymerase based expression system, was previously achieved by Esther Yu (Yu and Owttrim, 2000) and Sonya Kujat-Choy (Kujat and Owttrim, 2000), respectively. Each

construct, pRSET29(*crhC*) and pRSET:*crhR* (Table 2.2), contains an in-frame translational fusion of the respective gene's coding sequence for a His_6 tag at the amino terminus of the translated protein. *E. coli* JM109 transformed with either pRSET29(*crhC*) or pRSET:*crhR*, allows for overexpression of recombinant protein.

2.2.3.i Induction of His_6-CrhC and His_6-CrhR

E. coli JM109 (pRSET) harboring *crhC* or *crhR* was induced as described by (Yu and Owttrim 2000) and (Kujat and Owttrim 2000), respectively. Briefly, a single colony of *E. coli* JM109 (pRSET) was inoculated into 5 ml LB containing 100 μg/ml ampicillin, grown at 37°C overnight, and diluted 1:100 into 25 ml fresh LB/ampicillin (100 μg/ml) media. The diluted culture was grown at 37°C with shaking to mid log phase to OD_{600} = 0.6, and induced by addition of IPTG (Sigma, St Louis, MO, USA) and M13/T7 phage (provides T7 RNA polymerase for the overexpression) (Invitrogen, Burlington, ON, Canada) to a final concentration of 0.5 mM and 1 X 10^9 pfu/ml, respectively. For CrhC expression, cultures were transferred to 20°C and incubated overnight with continuous shaking. CrhR expression was performed by incubation of cultures for 2 hrs after induction at 37°C with continuous shaking. Harvested cells were stored as a pellet at -80°C.

2.2.3.ii Preparation and purification of soluble His_6-CrhC and His_6-CrhR protein

Induced frozen cells prepared as described in section 2.2.3.i were thawed on ice, resuspended in 3 ml lysis buffer I (50 mM NaH_2PO_4, pH 8.0; 300mM NaCl; 10 mM imidazole). Cells were broken by 4 cycles consisting of 1 min sonication on ice followed by 1 min ice water bath incubation between each sonication cycle, using a Braun-Sonic 2 000 sonicator fitted with a needle probe at 40 watts. The sonicated cells were centrifuged at 4°C for 30 min at 14 000 x g. The supernatant was incubated with 1 ml of a 50% Ni-NTA slurry (QIAGEN, Burlington, ON, Canada) prewashed with 3 X 3 ml lysis buffer and mixed gently by shaking at 4°C for 30 min. The lysate–Ni-NTA mixture was loaded onto a chromatograph column (Bio-Rad) and the flow-through collected. The column was washed 5 X with 4 ml wash buffer I (50 mM NaH_2PO_4, pH 8.0; 300 mM NaCl) containing 20 mM imidazole, and once with 0.5 ml of buffer II (buffer I; 40 mM imidazole). Protein elution was performed with 1 ml of buffer III (buffer I, 250 mM imidazole), protein concentration was determined by Bradford protein assay (Bio-Rad) and stored in aliquots in 50% (v/v) glycerol at –80°C.

2.2.4 Pull-down of proteins from *Anabaena* co-purifying with His_6-CrhC

His_6-CrhC was bound to a Ni-NTA column as described for the purification protocol given in section 2.2.3.ii, however, the His_6-CrhC was not eluted from the Ni-NTA column after washing with 40 mM imidazole. Cyanobacterial protein extract (500 µg/ml) prepared as described in section (2.2.1) was incubated with the Ni-NTA column-bound His_6-CrhC at 4°C for 1 h.

The flow through was collected and the column was washed 3 X with 3 ml of buffer I containing 20 mM imidazole. Bound protein was eluted with buffer III as described in section 2.2.3.ii. Protein fractions generated at different steps of the procedure were analyzed by 10% SDS-PAGE and visualized by Coomassie blue staining. The co-purifying proteins were excised from the gel and sent for mass spectrometry analysis (IBD, University of Alberta)

2.2.5 Removal of *E. coli* proteins co-purifying with His_6-CrhC

The use of His_6-CrhC in a co-immunoprecipitation experiment requires the preior removal of *E. coli* proteins interacting with His_6-CrhC. Therefore, induced *E. coli* frozen cells were prepared and lysed as described in section (2.2.3.ii) with one modification. Cells were resuspended in PBS-I containing 1% of the detergent octyl- D-glucoside (w/v). The octyl- D -glucoside was included to disrupt, in a non denaturing manner, the interaction of His_6-CrhC with any protein co-purifying with it from *E. coli*, and thus yielding a pure fraction of His_6-CrhC to be used in co-immunoprecipitation experiment. Cells were broken by sonication as described in section 2.2.3.ii. The sonicated cells were further incubated on ice for 30 min. The rest of the purification procedure proceeded as described in section 2.2.3.ii. The efficiency of the removal of proteins interacting with His_6-CrhC was tested by immunoblotting, and mass spectrometry.

2.2.6 His_6-CrhC analysis by MALDI-MS

The purity and molecular weight of the His_6-CrhC performed as described in section 2.2.5 was analyzed by MALDI-MS. The analysis was performed at the chemistry department, University of Alberta. The CrhC sample contained 110

ng/µl protein in 40 mM imidazole, 50 mM NaH_2PO_4, 0.3 M NaCl, pH 8.0, 50% glycerol. The sample was diluted 1:50 (v/v) with 0.1 % trifluoroacetic acid (TFA) to give a final glycerol concentration of 1% and a pH 4.0, 500 µl of which was aspirated only once for binding CrhC to the resin of the Zip Tip C4 column. The protein was eluted with 50% CH_3CN in 0.1% TFA through Zip Tip C4 and then analyzed by the MALDI/MS (Applied Biosystem Voyager System 108, Streetsville, ON, Canada).

2.2.7 Co-immunoprecipitation of proteins from *Anabaena* with His_6-CrhC

The purified His_6-CrhC (50 µg protein; 2.2.5) was incubated with clarified cyanobacterial protein extract produced either from cold shocked or warm grown cells (500 µg protein; section 2.2.1) for 1 hr at 4°C. Protein A-anti-CrhC antibody covalently coupled beads (200 µl bed volume) prepared as described in section 2.2.2.i were added to the lysate. The immunoprecipitation was performed as described in section (2.2.2.ii). The co-immunoprecipitated polypeptides were excised from a Coomassie blue stained gel and analyzed by mass spectrometry.

2.2.8 SDS-polyacrylamide gel electrophoresis

Electrophoresis was performed as described by Laemmli (1963). Briefly, protein samples were separated electrophoretically on 10% SDS-polyacrylamide gel (SDS-PAGE) using a Bio-Rad Mini-PROTEAN II electrophoresis cell. Resolving gels were made by combining 1.25 ml 30% (w/v) acrylamide: 0.8% (w/v) bisacrylamide (Bio-Rad, Burlington, ON, Canada); 450 µl 3 M Tris/HCl, pH 8.8; 37.5 µl 10% (w/v) SDS; 1.8 ml distilled H_2O; 1.875 µl 1.5% (w/v) ammonium persulfate (APS); 5 µl TEMED. The gel was poured allowing space

for a stacking gel of 1 cm. Immediately after pouring, isopropanol was layered on top of the acrylamide solution and the gel allowed to polymerize for 20 to 45 min. The isopropanol was poured off, and the gel surface washed thoroughly with dH_2O. The excess dH_2O was removed gently by blotting with Whatman 3M paper. A stacking gel was made by combining 0.8 ml 30% (w/v) acrylamide: 0.8% (w/v) bisacrylamide; 1.25 ml 0.5 M Tris, pH 6.8; 50 µl 10% (w/v) SDS; 2.675 ml distilled H_2O; 4 µl TEMED and 250 µl 1.5% (w/v) APS. After the insertion of a 10 well comb, the stacking gel was poured and allowed to polymerize for 30 min. The wells were rinsed with 1 X running buffer (25 mM Tris; 0.192 M glycine; 0.1% [w/v] SDS) prior to loading. Samples were prepared by adding 1/3 volume of loading buffer (125mm Tris pH 6.8; 4% [w/v] SDS; 20% [v/v] glycerol, 10% [v/v] β-mercaptoethanol and 0.02% bromophenol blue) and boiling for 5 min. The gels were electrophoresed in 1 X running buffer at 190 V. Either Kaleidoscope or pre-stained or unstained low protein molecular weight standards (Bio-Rad), treated identically to protein samples, were run on each gel.

2.2.9 Staining proteins in polyacrylamide gels

After electrophoresis, protein gels were stained with Coomassie brilliant blue in order to visualize polypeptides. Gels were fixed in a destaining solution of 30% (v/v) methanol, 10% (v/v) acetic acid for 10 min to remove SDS, and stained in a solution of 0.25% (w/v) Coomassie brilliant blue R250, 45% (v/v) methanol, 10 % (v/v) acetic acid for 10 min. The staining solution was removed and the gel destained by incubation in destaining solution.

2.2.10 MALDI-QTOF mass spectrometry analysis

A standard procedure was utilized by the Institute of Biomolecular Design (IBD) at the University of Alberta. Briefly, Coomassie-blue stained proteins were excised manually from a 10% SDS-PAGE gel and were digested with trypsin (Promega). Peptides were then extracted, and analyzed by MALDI-QTOF/MS.

2.2.11 Protein analysis and database Search

The identity of the proteins was resolved by searching by MALDI-QTOF/MS the entire database of the National Center for Biotechnology information (NCBI) among all species using http://www.matrixscience.com/cgi/search_form.pl (Mascot: MS/MS Ions Search) for homology of the peptide masses generated. The search parameters allowed for oxidation of methionine, carbamidomethylation of cysteine, one miscleavage of trypsin, and 30 ppm mass accuracy. The identification was repeated at least twice using proteins isolated from different gels.

2.2.12 Co-localization of CrhC with ribosomes

Immunogold labeling of cold shocked and warm cells were done as described in section 2.1.6. Serial ultrathin sections (80 nm) were mounted on copper grids. Grids were incubated in the indicated primary antibody (1:100 dilution). The secondary antibody used was a goat anti-rabbit IgG coupled to colloidal gold particles (Sigma, St Louis, MO, USA). For co-localization experiments, one side of the grid was probed with anti-CrhC (10-nm gold particles), and the other side was probed with anti-Rps1 (5-nm gold particles), the grids were examined, and the focus was adjusted to obtain an acceptable visibility

of both sizes of the gold particles (10 and 5 nm) within the fields examined and then captured on photographic film.

2.3 Phosphorylation profile of *Anabaena* in response to cold shock

2.3.1 Cold and salt shock conditions

Anabaena cultures were grown and cold shocked as described in section 2.1.1, Salt shocked cells were obtained as described by Apte and Bhagwat (1989) with slight modification. Briefly, cultures in the mid-log phase were harvested, suspended in fresh BG-11 medium and grown overnight at 30°C before the addition of NaCl (100 mM). After treatment, cells were harvested by centrifugation at 11 000 x *g* for 15 minutes at the appropriate growth temperature, the pellet was flash frozen in liquid nitrogen, and stored at -80°C.

2.3.2 Cyanobacterial protein extraction for *in vitro* phosphorylation

Spheroplasts from cold and salt shocked cells were prepared as described in section (2.1.3). Spheroplasts were lysed mechanically by vortexing in an equal volume of glass beads (0.2 to 0.3 mm) (Impandex, Clifton, NJ, USA), centrifuged at 1000 x *g* to remove unbroken spheroplasts, and the supernatant clarified by centrifugation at 14 000 x *g* for 30 minutes at 4°C. The clarified supernatant (cell-free extract) was removed and its protein concentration determined using a Bradford assay with BSA as the standard.

2.3.3 *In vitro* phosphorylation

In vitro phosphorylation was performed as described by Hong *et al.* (1993). The reaction mixture contained 50 mM Tris-HCL (pH 7.4), 50 mM NaCl,

10 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 5 μCi [γ-^{32}P]ATP (AmershamBiosciences), and 10 μg cell free extract. Reactions were incubated at room temperature for 5 min, quenched by the addition of 3 X Laemmli sample buffer, boiled, and separated by a 10% SDS-PAGE. Proteins were transferred to nitrocellulose membranes and phosphoproteins detected using either autoradiography or a phosphor screen (Molecular Dynamics Model 445 S1, GE health care, Beverly, MA, USA).

2.3.4 Immunodetection of phosphorylated proteins and EF-Tu

Western blot analysis of proteins isolated from cold and salt shocked *Anabaena* cells was performed as described in section (2.1.2). Briefly, proteins extracted from cold or salt stressed *Anabaena* at the indicated time and treatment were separated by 10% SDS-PAGE (20 μg protein/lane) and transferred to nitrocellulose membranes (Hybond ECL, AmershamPharmaciaBiotech), using a semidry apparatus (Tyler Research, Canada). Western blots were probed with either polyclonal (CellSignal, Beverly, MA, USA) or monoclonal (QIAGEN) anti-phosphothreonine, or monoclonal anti-phosphoserine (QIAGEN) or anti-phosphotyrosine (CellSignal) anti-sera at 1:200 dilution. The washing and blocking steps were performed according to manufacturers' recommendations. After washing, the blots were incubated with the appropriate horseradish peroxidase-linked secondary antibody (AmershamBiosciences) at a dilution of 1:1000 for 60 minutes. For EF-Tu immunodetection, membranes were blocked with 5% non-fat powdered milk in Tris-buffered saline (TBS, pH 8.0) and incubated with anti-EF-Tu antibodies (T. Kannan, University of Texas) at 1:5000

dilution for 1 hour. Blots were washed for 5 min each consecutively with TBS, TBST (TBS with 0.1% Tween 20), and TBS, incubated with secondary antibody linked to horseradish peroxidase (Sigma) at 1:20 000 dilution for 20 min. Blots were visualized by chemiluminescence (ECL, AmershamBiosciences) according to the manufacturers' specifications.

2.3.5 Isolation of phosphorylated proteins

Anabaena were cold stressed for 0.25 h and mechanically lysed in the presence of CHAPS (0.25% w/v) as recommended by the PhosphoProtein Purification Kit (Qiagen). The lysate was clarified by centrifugation at 11 000 x *g* for 30 minutes at 4°C, and its protein concentration determined. An aliquot of the clarified lysate was treated with alkaline phosphatase (2.5 U/20 µg protein) (Roche) for 10 minutes at room temperature and the reaction quenched by addition of alkaline phosphatase inhibitor (Roche). The protein concentration of each aliquot (with or without alkaline phosphatase treatment) was adjusted to 0.1 mg/ml before loading on the PhosphoProtein Purification column (QIAGEN). The column was washed and eluted as described by the manufacturer with the third elution fraction containing the highest protein yield, which was further concentrated on a Nanosep ultrafiltration column (Millipore). The protein fractions thus obtained were separated by 10% SDS-PAGE and the gel was stained with Coomassie blue to visualize eluted proteins. Mass spectrometry analysis was performed on all eluted proteins as described in section (2.2.10).

2.3.6 Database Search

Proteins were identified based on the highest ranking obtained by a search of the entire National Center for Biotechnology information (NCBI) database using Mascot: MS/MS Ions Search **http://www.matrixscience.com/cgi/search_form.pl**. The search parameters allowed for oxidation of methionine, carbamidomethylation of cysteine, one miscleavage of trypsin, and 30 ppm mass accuracy. The identification was repeated at least once using proteins purified from different experiments. The identified fragments were further analyzed for post-translational modifications utilizing FindMod (www.expasy.org/Findmod) as described by Wilkins *et al.*, (1999).

Chapter 3

Polar-biased localization of the cold shock-induced RNA helicase, CrhC, in the cyanobacterium *Anabaena* sp. strain PCC 7120.

Parts of this chapter have been published in Molecular Microbiology. 50(4): 1439-1446, 2003. Also, selected EM images of this chapter were presented on the cover of the same issue.

3.1 Introduction

RNA helicases are generally believed to unwind double-stranded RNA using energy derived from nucleoside triphosphate (NTP) hydrolysis functioning as ATP-driven translocating machines or RNA-protein dissociation factors (Linder *et al.*, 2001; Schwer, 2001). This activity is potentially required for any process involving RNA metabolism (Staley and Guthrie, 1998; de la Cruz *et al.*, 1999; Linder and Daugeron, 2000). RNA helicases are characterized by an evolutionarily conserved core helicase domain flanked by non-conserved amino acid domains proposed to determine substrate specificity, interaction with other cellular components, additional enzymatic activities, and/or subcellular localization (de la Cruz *et al.*, 1999; Tanner and Linder, 2001).

Recently, the subcellular localization of a variety of eukaryotic RNA helicases has been obtained using a combination of fluorescence microscopy localization of GFP-helicase fusion proteins, immunogold or immunofluorescence microscopy, and cell fractionation. For example, subcellular localization has been an aid in determining function in various nuclear bodies (Schmitt *et al.*, 1999; Staley and Guthrie 1998; Zirwes *et al.*, 2000; Bléoo *et al.*, 2001; Snay-Hodge *et al.*, 1998; Tang *et al.*, 1997). RNA helicases have also been localized to mitochondria (Séraphin *et al.*, 1989) and chloroplasts (Owttrim *et al.*, 1994). Less advanced is our understanding of RNA helicase localization in prokaryotes. In *E. coli* RhlB is associated with the RNA degradosome (Py *et al.*, 1996) while CsdA is ribosome-associated in cold-shocked cells (Jones *et al.*, 1996). RNA helicase association with various membranes has also been reported, for example RhlB

(Liou *et al.*, 2001), all RNA helicase-like (HEL) proteins encoded by positive-strand RNA viruses (van der Heijden *et al.*, 2001; Restrepo-Hartwig and Ahlquist, 1996), and SecA in eubacteria (Schmidt *et al.*, 2001).

We have previously reported the characterization of an environmentally regulated RNA helicase gene, *crhC*, from the cyanobacterium *Anabaena* sp. strain PCC 7120. CrhC is a member of the DEAD-box family of RNA helicases; however, the highly conserved SAT box is altered to FAT (Chamot *et al.*, 1999). CrhC expression is tightly regulated by temperature with both transcript and protein accumulation only observed at temperatures below 30˚C (Chamot *et al.*, 1999; Chamot and Owttrim, 2000). CrhC therefore belongs to a group of proteins whose expression is induced at low temperature, the cold shock proteins (CSPs). Expression of two other RNA helicases are also known to be induced by low temperature, CsdA in *E. coli* (Jones *et al.*, 1996; formerly termed DeaD by Toone *et al.*, 1991) and DeaD in the archaeon *Methanococcoides burtonii* (Lim *et al.*, 2000). Biochemically, CrhC exhibits RNA-dependent ATPase and dsRNA unwinding activity which is (d)ATP-dependent and unidirectional, progressing in the 5′ to 3′ direction (Yu and Owttrim, 2000). Based on our expression and biochemical data, we have proposed that CrhC may perform a role in cyanobacterial cold acclimation by actively unwinding the stable 5′ UTR's characteristic of CSP mRNAs (Chamot and Owttrim, 2000; Yu and Owttrim, 2000).

In addition to CrhC, cyanobacteria respond to cold shock by inducing the expression of a number of CSPs, including fatty acid desaturases (Los *et al.*,

1997), RNA chaperones (Sato, 1995), ribosomal proteins (Sato *et al.*, 1997), heat shock proteins (Porankiewicz *et al.*, 1998), NADH dehydrogenase subunits, sigma 70, and EF-G (Suzuki *et al.*, 2001). In particular, the fatty acid desaturases (Mustardy *et al.*, 1996) and NADH dehydrogenase subunits are membrane-associated. The mechanism(s) by which these proteins are localized to the membrane during cold shock is, however, not known.

Recently, two-component signal transduction systems have been implicated in cold sensing and response in prokaryotes (Aguilar *et al.*, 2001; Suzuki *et al.*, 2001). The temperature sensor histidine kinase in these systems is an integral plasma membrane protein. A variety of plasma membrane-associated histidine kinases which function as environmental sensors in prokaryotes are polar localized (Lybarger and Maddock, 2001). In fact, asymmetrically localized proteins associated with the bacterial plasma membrane are required for a growing list of essential cell processes including host-pathogen interactions, cell motility, chromosome replication and segregation, cell wall formation, and cell division and cell cycle control (Shapiro and Losick, 2000; Lybarger and Maddock, 2001; Shapiro *et al.*, 2002). Although the mechanism(s) by which protein polarity is established are not fully understood, one hypothesis suggests direct insertion into the polar septum via co-transcriptional translation and protein translocation (transertion) (Woldringh, 2002). Here, we report that the cold shock-induced RNA helicase, CrhC, is a plasma membrane-associated polar-localized protein in cyanobacteria. Since major cold shock-induced changes in physiology occur at the plasma membrane, we propose that CrhC is involved in the

transertion of proteins into or across the plasma membrane during cyanobacterial acclimation to cold shock.

3.2 Results

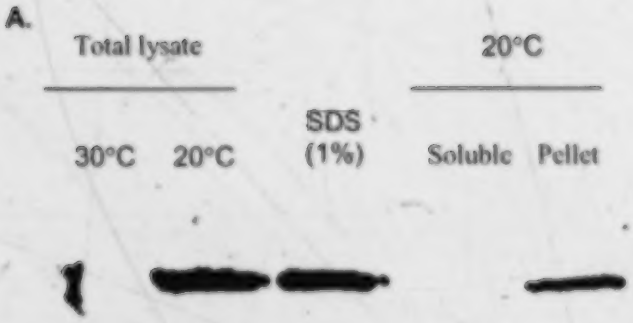
3.2.1 CrhC is membrane associated in cold-shocked Anabaena cells

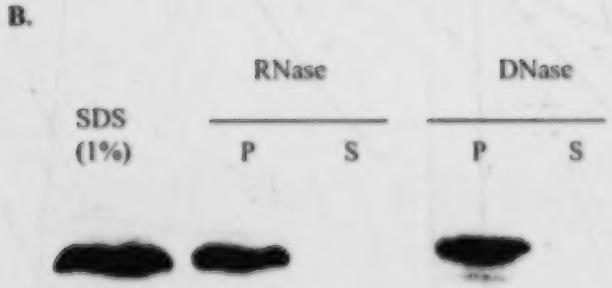
Since it is well established that *crhC* transcript and protein expression is limited to temperatures below 30°C, it was of interest to determine the subcellular localization of CrhC during cold shock. Optimization of immunoblot procedures revealed that CrhC was consistently detected in the pellet fraction obtained after centrifugal clarification of cell lysates. Therefore the accumulation and solubility of CrhC was examined in cells grown at warm (30°C) or cold shock (20°C) temperatures. Immunoblot analysis confirmed that CrhC was only expressed in *Anabaena* cells subjected to cold shock (20°C) and not in cells grown at a temperature which does not promote the cold shock induction of CrhC expression (30°C) (Fig. 3.1.A). Centrifugation of these lysates, yielding crude soluble and pellet fractions, indicated that CrhC is only detectable in the pellet fraction. CrhC was solublized by treatment of the pellet fraction with SDS (1%) (Fig. 3.1.A). The possibility that CrhC was associated with the pellet fraction by an interaction with nucleic acids was investigated. Neither RNase nor DNase treatment released CrhC from the pellet fraction obtained from cold shocked *Anabaena* cells (Fig. 3.1.B). These results suggest that CrhC is membrane associated.

Fig. 3.1. Immunoblot analysis of CrhC solubility.

A. CrhC solubility. Reading left to right across the panel the lanes contained: total lysate from *Anabaena* cells grown at 30°C for 6 h; total lysate from *Anabaena* cells grown at 20°C for 6 h; supernatant obtained after treatment of the pellet fraction from cold shocked cells with SDS (1%); soluble fraction from cells grown at 20°C for 6 h; pellet fraction from cells grown at 20°C for 6 h.

B. CrhC is not insoluble as a result of association with nucleic acids. All fractions were obtained from cells grown at 20°C for 6 h. Reading left to right across the panel the lanes contained: the supernatant obtained after SDS (1%) treatment of the pellet fraction; pellet (P) and supernatant (S) fractions obtained after RNase A (5 U) treatment of the pellet fraction; pellet (P) and supernatant (S) fractions obtained after DNase I (3 U) treatment of the pellet fraction. Nuclease treatments were performed at 37°C for 20 min.


A.
Total lysate
20°C
SDS
(1%)
30°C
20°C
Soluble
Pellet


B.
RNase
DNase
SDS
(1%)
P
S
P
S

3.2.2 CrhC is associated with the plasma membrane

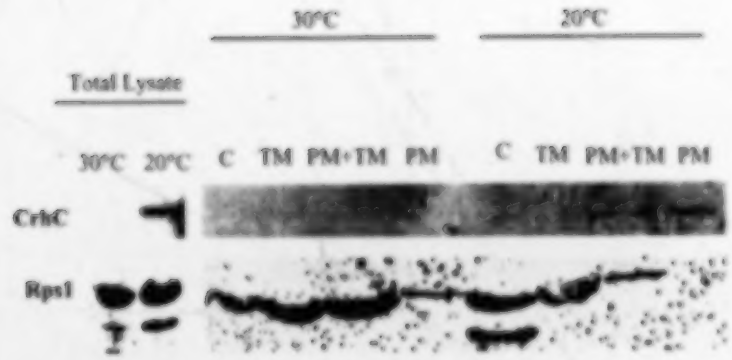
To identify the subcellular fraction with which CrhC associates, cellular membranes were purified from cold shocked *Anabaena* cells using a standard discontinuous sucrose gradient procedure (Murata and Omata, 1988). Immunoblot analysis of the subcellular fractions obtained from cells grown at 20°C and 30°C is shown in Fig. 3.2. Lysates obtained from cells grown under the two conditions before ultracentrifugation are shown as a reference. CrhC was detected in the plasma membrane (PM) and the mixture of plasma membrane plus thylakoid membrane (PM+TM) fractions of cold shocked cells (Fig. 3.2). CrhC was not detected in any membrane or cytoplasmic fraction obtained from cells grown at 30°C (Fig. 3.2). These results confirmed both the cold expression and plasma membrane-specific localization of CrhC. As a control, the distribution of ribosomal protein S1 (Rps1) was also analyzed in the subcellular fractions. The localization pattern of Rps1 was distinctly different from that observed for CrhC (Fig. 3.2). Rps1 was equally distributed between the cytoplasmic and thylakoid membrane fractions of cells grown at both temperatures. This distribution is in agreement with reports indicating ribosomes, and thus Rps1, are localized to both the cytoplasm (Sugita *et al.*, 2000) and thylakoid membranes (Tyystjarvi *et al.*, 2001) in cyanobacteria.

3.2.3 CrhC is an integral plasma membrane protein

To examine whether CrhC is an integral membrane protein, pellet fractions obtained after lysis of spheroplasts produced from cold shocked *Anabaena* cells were treated with detergents which differentially disrupt integral

Fig. 3.2. Subcellular localization of CrhC.

Subcellular fractions were obtained by sucrose density centrifugation of lysates from *Anabaena* cells grown at 30°C or at 20°C for 6h, as described in the materials and methods. Aliquots (5 µg protein) were subjected to immunoblot analysis in duplicate, using anti-CrhC antiserum or anti-Rps1 antiserum (1:5 000) as indicated. PM, plasma membrane; TM, thylakoid membrane; C, cytoplasm. As a control, total cell lysates (20 µg protein) obtained from cells grown at the respective temperatures are shown on the left.


30°C
20°C
Total Lysate
30°C 20°C
C TM PM+TM PM
C TM PM+TM PM
CrhC
Rps1

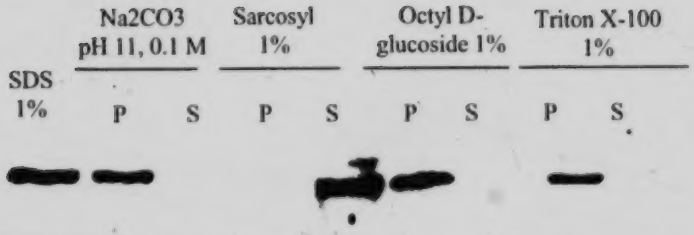
membrane protein-lipid interactions. Immunoblot analysis revealed that CrhC was solubilized by treatment with the anionic detergents SDS (1 %) and Sarkosyl (1 %). Although not visible in the Figure, CrhC was partially released from the pellet fraction by the nondenaturing Zwitterionic detergent CHAPS (1 %), and the nonionic detergents octyl-D-glucoside (1 %), Triton X-100 (1 %), and Triton X-114 (1 %), but remained insoluble in the presence of the nonionic detergent Brij 35 (1%) (Fig. 3.3). CrhC also remained insoluble after treatment of the pellets with washes (0.1M Na_2CO_3 pH 11 and 1M NaCl) known to disrupt hydrophobic interactions characteristic of peripheral membrane proteins. Solubilization by anionic detergents capable of complete disruption of lipid-protein interactions and not by treatments that disrupt hydrophobic interactions is consistent with CrhC being an integral membrane protein.

3.2.4 CrhC is associated with the cytoplasmic face of the plasma membrane

Trypsin treatment of spheroplasts isolated from cold shocked *Anabaena* cells was utilized to determine CrhC orientation in the plasma membrane. CrhC has several trypsin cleavage sites (Figure 3.4) and is completely degraded after 5 min of direct contact with trypsin (data not shown). The result obtained from a single blot probed consecutively with a series of antibodies is shown in Figure 3.5. The blot contained total extracts obtained from spheroplasts of cold shocked *Anabaena* cells treated with trypsin for 0, 5, 10, and 15 min. There was no significant change in CrhC levels as a result of trypsin digestion over the time course of the experiment (Fig. 3.4, CrhC). Trypsin treatment also did not affect

Fig. 3.3 Membrane association of CrhC.

The pellet fraction obtained from *Anabaena* cells grown at 20°C for 6 h was treated with the indicated reagents for 30 min. All buffers contained EDTA (5mM). P and S represent the pellet and supernatant fractions were subjected to immunoblot analysis and CrhC detected with anti-CrhC antiserum. The supernatant obtained after SDS (1 %) treatment of the pellet fraction is included as a reference.


Na2CO3
pH 11, 0.1 M
Sarcosyl
1%
Octyl D-
glucoside 1%
Triton X-100
1%
SDS
1%
P
S
P
S
P
S
P
S

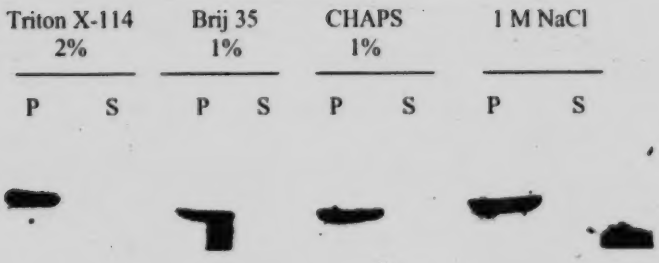

Triton X-114
2%
Brij 35
1%
CHAPS
1%
1 M NaCl
P
S
P
S
P
S
P
S

Fig 3.4. CrhC trypsin cleavage sites

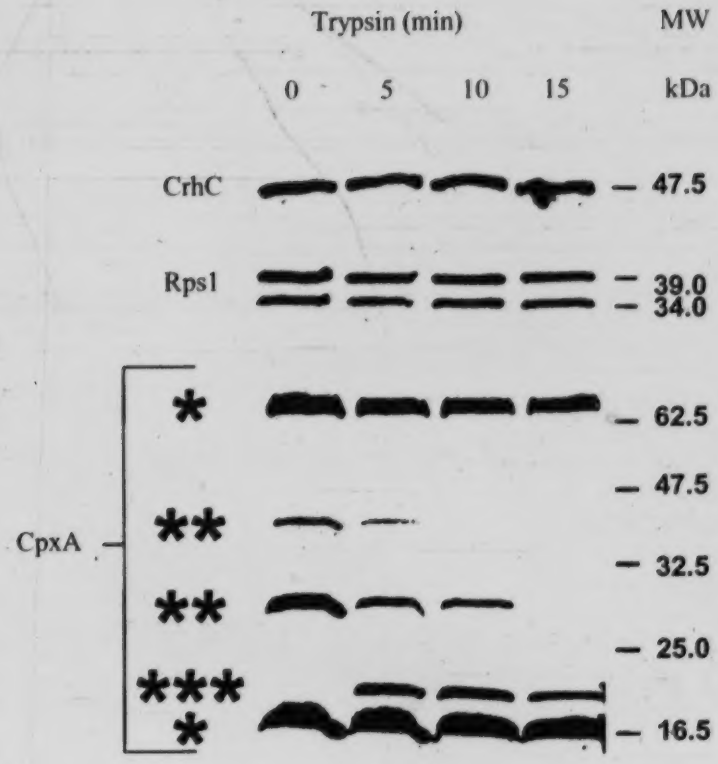
CrhC amino acid sequence with trypsin cleavage sites at peptide bonds involving lysine (k) or arginine (r) labeled with a star (*).

1 msfshlglsn eiinavtelg ytk*ptpiqmq **30**
31sipavlsgr*d llr*gaqtgtg k*tasftlpll **60**
61hyypk*ivlk*a lqcfltir*al iltptr*elaa **90**
91qvessvr*dyg k*ylk*lnsmvm fggvsinpqk* **120**
121qr*lk*gr*vdil vatpgr*lldh vqqgtvnlsq **150**
151ieilvldead r*mldmgfir*d ir*r*ilsllpk **180**
181qr*qnllffat fsdk*ik*elaa gllnr*pqmie **210**
211var*r*nvtadt vtqk*vyk*ier* dr*k*r*dllahl **240**
241ir*k*dnwyqvl vftr*tk*ygad r*lvk*qlgher* **270**
271iqalaihgnk* sqsar*thala k*fk*ngslqvl **300**
301vatdiaar*gl diselpyvvn fdlpyvpedy **330**
331vhr*igr*tgr*a gasgeavslv sadeyhllad **360**
361iek*lik*er*lp felvagigan sqak*peptqd **400**
401erkqkpkdsq hqprsaaqgv pk*k*sgk*k*r*lt **430**
431 nsgk*r* **435**

the levl of either of the two cytoplasmic isoforms of Rps1 present in cold shocked *Anabaena* cells (Fig. 3.4, Rps1). These results indicate that CrhC and Rps1 were not accessible to trypsin degradation under the conditions utilized in this experiment. As a control for a protein that spans the plasma membrane, the blot was also probed with a polyclonal antibody against the *E. coli* sensor histidine kinase, CpxA. CpxA is located in the plasma membrane and the anti-CpxA antibody detects a number of soluble and insoluble polypeptides in *E. coli* cell extracts (T. Raivio, personal communication). The CpxA antibody reacted with four (4) polypeptides in *Anabaena* cell extracts (Fig. 3.4, CpxA 0 time). Trypsin treatment of *Anabaena* spheroplasts affected these polypeptides differentially. The results indicate that the CpxA antibody detects two soluble (17 and 65 kDa) trypsin-inaccessible (Fig. 3.4, CpxA star) and two membrane-associated (31 and 41 kDa) trypsin-accessible (Fig. 3.4, CpxA double star) histidine kinase-related polypeptides in *Anabaena*. A fifth, 20 kDa polypeptide was detected as a result of trypsin treatment (Fig. 3.4, CpxA triple star) which most likely represents a stable breakdown product of one of the trypsin-accessible polypeptides. The solubility of the four histidine kinase-related proteins was confirmed by cell fractionation (data not shown). Thus, CrhC responds to trypsin treatment in a manner similar to that observed for the cytoplasmic proteins Rps1 and the 17 and 65 kDa histidine kinases thereby indicating that CrhC is localized to the cytoplasmic region. This result, combined with its plasma membrane-association indicates that CrhC is oriented towards the cytoplasm.

Fig. 3.5. CrhC orientation in the plasma membrane.

Spheroplasts produced from *Anabaena* cells grown at 20°C for 6 h were treated with trypsin (15 μg/ml) for 5, 10, and 15 min. Trypsin treatment was halted by the addition of trypsin inhibitor (40 μg/ml) and EDTA (5mM) at the indicated times. The spheroplasts were centrifuged and total lysates (5 μg protein) analyzed by SDS-PAGE, transferred to nitrocellulose and histidine kinases related to the *E. coli* CpxA protein detected using anti-CpxA antibody. In the CpxA panel, proteins which are not accessible to trypsin degradation (17 and 65 kDa) are indicated by a •, proteins which are trypsin-accessible (31 and 41 kDa) by ••, and a polypeptide which appears only during trypsin treatment (20 kDa) by •••. The same blot was stripped and CrhC detected with anti-CrhC antiserum. The blot was stripped again and Rps1 detected using anti-Rps1 antiserum.


Trypsin (min)
MW
0
5
10
15
kDa
CrhC
47.5
Rps1
39.0
34.0
CpxA
*
62.5
47.5
**
32.5
**
25.0
***
*
16.5

3.2.5 Computer analysis does not predict a transmembrane or a lipid anchor attachment domain in CrhC

Since the results suggest that CrhC is an integral membrane protein, it was of interest to determine the mechanism by which CrhC is associated with the plasma membrane. The amino acid sequence of CrhC was analyzed with the protein topology predicting programs PepTool 1.1 (BioTools Inc., Edmonton, Canada), DAS transmembrane prediction server (Cserzo *et al.*, 1997), and PSORT (psort.nibb.ac.jp). Each program produced similar results indicating that CrhC does not posses a potential transmembrane domain (data not shown). Cyanobacteria also possess a number of lipoproteins anchored to either the outer portion of the plasma membrane or facing into the thylakoid lumen via a lipid anchor (Li *et al.*, 2002). Lipoproteins possess both an N-terminal leader sequence followed by a lipobox, specified by the amino acid sequences CQPQ or CASA, with the Cys residue being the lipid attachment site. The lipoprotein search programs PSORT and DOLOP (mrc-lmb.cam.ac.uk/genomes/dolop/analysis.htm) did not detect a putative lipobox in the CrhC sequence. While the signal peptide prediction program PSORT did not detect a possible N-terminal signal sequence, SignalP V1.1 (cbs.dtu.dk/services/SignalP), did predict a possible cleavage site between amino acids 89 and 90. Since, this cleavage would remove the first two conserved amino acid domains characteristic of RNA helicases, we do not believe this is a functional signal sequence. Taken together, these results indicate that neither a typical transmembrane domain nor a lipid anchor are involved in CrhC association with the *Anabaena* plasma membrane. However, CrhC being associated with the plasma membrane through a carbohydrate anchor cannot be excluded.

3.2.6 CrhC released from the plasma membrane by phospholipase C treatment

The possibility that a PI anchor anchors CrhC to the plasma membrane was investigated by treating plasma membrane fractions from cold shocked *Anabaena* with phospholipases. A variety of phospholipases with different specificity in cleaving PI anchors were utilized including PI specific Phospholipase C, Phospholipase C III, Phospholipase C IX, and Phospholipase D (Fig 3.6). CrhC was only released from purified PM preparations by phospholipase C III and PI specific phospholipase C (Fig 3. 6). The solubolized CrhC immuno-reactive polypeptide migrated with an apparent molecular weight 3-5 kDa lower than SDS- solubolized CrhC (Fig 3.6). The PI specific phospholipase C treatment of GPI anchored proteins results in the exposure of inisitol 1,2-cyclic monophosphate, which is the major epitope involved in the recognition of the cross-reacting determinant (CRD). Therefore, the supernatant and pellet fractions resulting from the PI specific phospholipase C digested plasma membranes from cold shocked *Anabaena* were probed with anti-CRD antibodies as the primary antibody (Fig 3.7A), and anti-CrhC antibodies (Fig 3.7B). The reaction with anti-CRD antibodies observed by Western blotting revealed a pattern similar to that with anti-CrhC antibodies (Fig 3.7B), PI specific phospholipase C solubilized porcine membrane dipeptidase (MDP) served as a positive control (Fig 3.7). Despite the positive results we have observed with the anti-CRD antibodies, we performed mild acid treatment on the supernatant resulting after digesting the PM with PI specific phospholipase C (Fig 3.7C). Mild acid treatment results in the destruction of the CRD epitope, where the inisitol 1,2-cyclic monophosphate is either deaminated by HNO_2 or decyclised by the 1 M HCl treatment. After HNO_2 and HCL treatment, there was no reaction with anti-CRD

Fig 3.6. Polypeptide analysis of phospholipase treatment of cold shocked plasma membrane fractions. The pellet (P), and supernatant (S) from 6 h cold shocked cells after treatment with 1% SDS, Phospholipase III, Phospholipase C IX, Phospholipase D, PI specific Phosphlipase C, blots were probed with anti-CrhC antibodies.

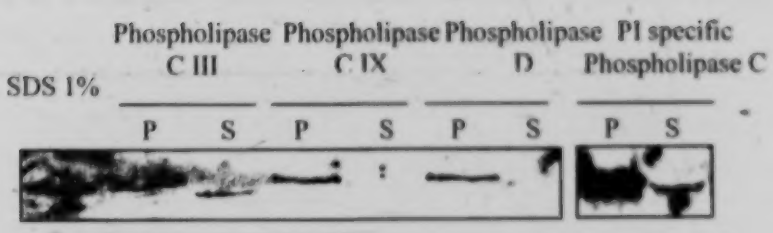
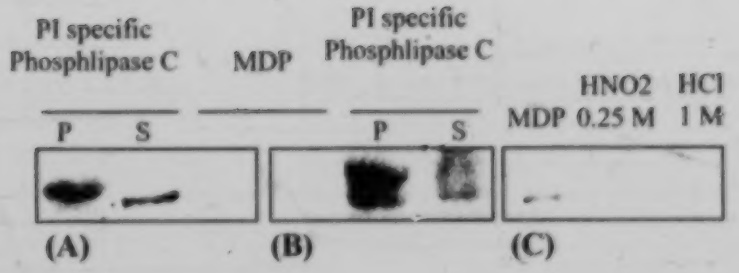

Phospholipase C III
Phospholipase C IX
Phospholipase D
PI specific Phospholipase C
SDS 1%
P S P S P S P S

Fig 3.7. Polypeptide blot analysis of plasma membrane after PI-specific phospholipase C treatment. The pellet (P) and the supernatant (S) resulting from the treatment probed with (A) anti-CRD antibodies, and (B) anti-CrhC antibodies. (C) The PI-specific phospholipase C treatment resulting supernatant was first treated with 0.25 M HNO2 and 1M HCL, the blot was probed with anti-CRD antibodies. MDP served as a control.


PI specific
Phosphlipase C
P
S
(A)
MDP
(B)
PI specific
Phosphlipase C
P
S
HNO2
MDP 0.25 M
HCl
1 M
(C)

antibodies, indicating that the mild acid treatment resulted in the destruction of the CRD epitope (Fig 3.7C). Despite that these results suggest that a PI or PI like anchor CrhC to the PM, the phospholipase treatment did not fully release CrhC from the membrane which may suggest that CrhC is associated with the plasma membrane through a carbohydrate-lipid anchor.

3.2.7 CrhC exhibits polar-biased localization

Subcellular localization of CrhC was further investigated using immunoelectron microscopy (IEM) analysis of ultrathin sections of *Anabaena* cells grown at 30°C or 20°C. CrhC localization was quantified by determining the number of gold particles per μm^2 of cell area (Table 3.1).

This analysis revealed that CrhC was 3.5-fold more frequently associated with the plasma membrane at the cell pole than on the lateral portion of the membrane. The same analysis indicated CrhC was over 1 000-fold more frequently observed at the cell pole than in the cytoplasm. The polar-biased localization and temperature regulated expression of CrhC is evident in the IEM images shown in Figure 3.8. As expected from previous studies, CrhC was not expressed in cells grown at 30°C, as only background labelling of gold particles was observed in these sections (Fig. 3.8A). A series of images obtained from different cells subjected to cold shock at 20°C for 6 h are shown in Fig. 3.8 B - H. Similar results were obtained with cells subjected to cold shock for up to 24 h (data not shown). Gold particle labelling, indicative of CrhC expression, was observed in sections obtained from cells grown under temperature shock conditions (20°C) (Fig. 3.8, B-H). Polar-biased localization to the plasma membrane at the septa between adjacent cells is

Table 3.1. Subcellular localization of CrhC in cold-shocked *Anabaena* cells.[a]

Location	Total no. of gold particles	Particles / μm^2 (mean ± SD)	Ratio Polar PM / other locations
Polar PM	506	283 ± 41[b]	1.0
Lateral PM	327	81 ± 31	3.5
TM + CPM	212	0.23 ± 0.17	1230.4

a. Cells were subjected to cold shock at 20°C for 6 h. A total of 62 longitudinal sections of cells within filaments were scored.

b. Value takes into account two plasma membranes per septum.

PM, plasma membrane; TM, thylakoid membrane; CPM, centroplasm.

Gold particles were manually counted in the plasma membrane for each longitudinal section. The numbers of gold particles per μm^2 were calculated as follows: The numbers of gold particles were enumerated manually in the parameters (length and width) of the area of either the lateral PM, polar PM and TM + CPM in photographic images of ultrathin sections (80 µm thickness/section) of various magnifications ranging from 20 000 to 80 000 times. The polar PM area is composed of two PM membranes of two adjacent cells; thereby the number of gold particles was scored within the width of the two plasma membranes in sections of cells within a filament and thus divided by a factor of two.

evident (Fig. 3.8, B-H). A higher magnification of a typical septum is shown in Fig. 3.8.G. CrhC is clearly associated with the plasma membrane at the cell poles between the two adjacent cells. As cell division involves the formation of two new plasma membranes and a cell wall at the division plane within the mother cell, it was of interest to determine if CrhC accumulated at these sites. This was indeed the case, as CrhC was observed to accumulate on the plasma membranes forming at the division plane within a dividing cell (Fig. 3.8.H). Since *Anabaena* grows as long filaments of individual cells, CrhC distribution along a cell filament was also examined. A representative IEM showing CrhC distribution within an *Anabaena* cell filament composed of four cells and three septa is shown in Fig. 3.9.A. The distribution of gold particles in this filament confirms the plasma membrane-association with a bias to the cell poles between adjacent cells.

IEM was also performed using antibodies raised against *E. coli* Rps1 (Fig. 3.9.B and C). Rabbit anti-Rps1 antiserum was used as a control for cytoplasmic versus membrane specific localization, as well as, the potential for non-specific association of rabbit antiserum with *Anabaena* septa. The labelling pattern obtained with anti-Rps1 antibodies was distinctly different from that obtained with the anti-CrhC antiserum. Rps1 was localized to the cytoplasmic region with no specific association of rabbit antibodies with the *Anabaena* plasma membrane or cell poles detected (Fig. 3.9.B and C).

Fig. 3.8. IEM subcellular localization of CrhC in cold shocked *Anabaena*.

CrhC was detected in ultrathin sections of *Anabaena* cells grown at 30°C (A); or at 20°C for 6 h (B-H) using anti-CrhC polyclonal antiserum at 1:100 dilution. Arrows indicate location of new septum formation. Bar = 500 nm (A-F, H) or 100 nm (G).

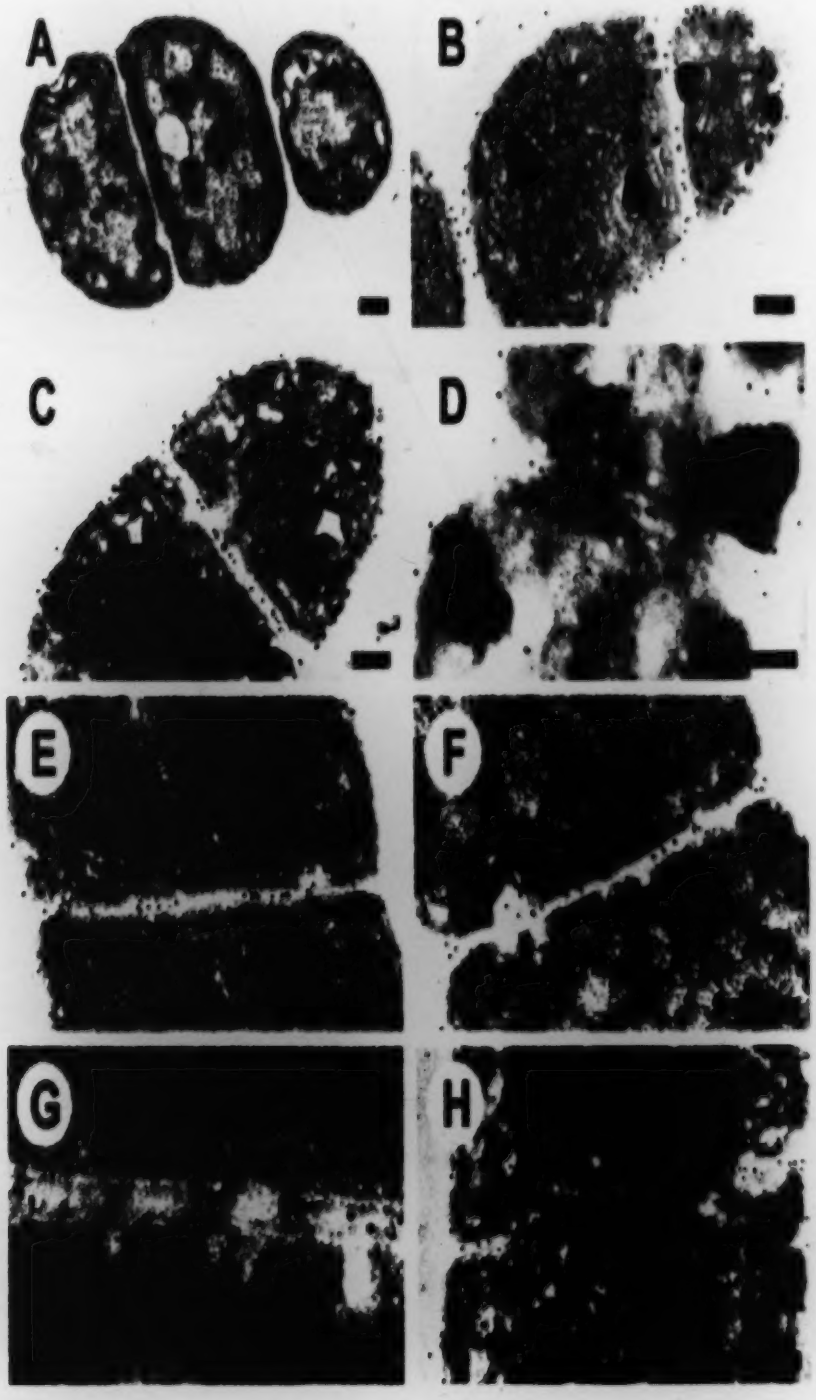
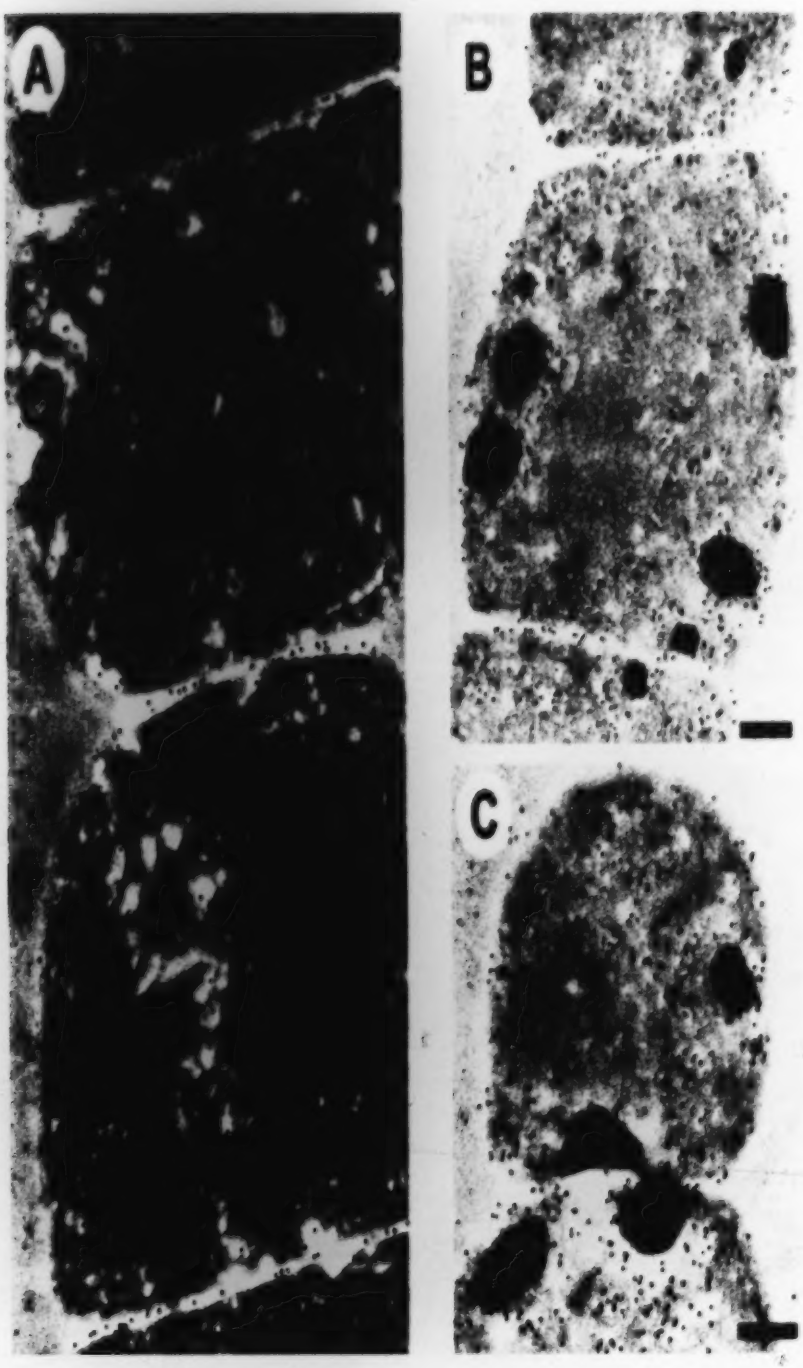

A
B
C
D
E
F
G
H

Fig. 3.9. IEM analysis of cold-shocked *Anabaena*.

A. CrhC distribution within an ultrathin section of an *Anabaena* filament consisting of four cells and three septa using anti-CrhC polyclonal antiserum at 1:100 dilution. Cells were grown at 20°C for 6 h. Bar = 500 nm.

B and C. As a control, ribosomal protein S1 (Rps1) was detected in ultrathin sections of *Anabaena* cells grown at 20°C for 6 h. Anti-Rps1 polyclonal antiserum was used at 1:100 dilution. Bar = 500 nm.


A
B
C

3.3 Discussion

This study reports the subcellular localization of the cold shock RNA helicase, CrhC, in the cyanobacterium *Anabaena* sp. strain PCC 7120. CrhC is an integral membrane protein associated with the cytoplasmic face of the plasma membrane. CrhC is not randomly distributed within the plasma membrane but is 3.5-fold more prevalent at the cell poles, accumulating at the septa between adjacent cells within filaments. CrhC is also associated with new cell poles forming within dividing cells. Although a variety of RNA helicases have been demonstrated to be membrane associated, none appear to be involved in the response to environmental shock (van der Heijden *et al.*, 2001; Restrepo-Hartwig and Ahlquist, 1996; Liou *et al.*, 2001; Schmidt *et al.*, 2001). Taken together with our results, the data provide evidence that various aspects of RNA metabolism catalyzed by RNA helicases occur at the plasma membrane in prokaryotes. CrhC must therefore perform a role in RNA metabolism that is concurrently associated with the plasma membrane, is more prevalent at the cell poles, and required during cold acclimation in cyanobacteria.

The relevance of asymmetrically localized proteins for cell growth and division and the sensing and response to environmental change has become increasingly evident in prokaryotes. CrhC distribution appears to be unique in comparison to other membrane-associated proteins. Although CrhC is more prevalent on the plasma membrane at the cell poles, it also associates with the lateral portion of the membrane. In *E. coli*, the RNA helicase RhlB, which is plasma membrane-associated through its incorporation into the RNA degradosome is localized predominately to regions adjacent to the plasma membrane with no evidence of polar bias (Liou *et al.*, 2001). Furthermore, CrhC is

uniformly distributed across the polar membrane in the septal region, with no clustering of particles evident. In contrast, various subunits of the chemoreceptor complex in *E. coli* (Lybarger and Maddock, 2000) or the DNA transfer protein VirD4 in *Agrobacterium* (Kumar and Das, 2002) are present in clusters at the cell poles. In addition, polar localization has not been observed for other cyanobacterial membrane-associated proteins, for example carbonic anhydrase (Soltes-Rak *et al.*, 1997) or a carotenoid-binding protein (Reddy *et al.*, 1989) both of which have a uniform distribution over the membrane surface with no obvious bias to the cell poles. Since CrhC does not exhibit polar clustering and can be detected in the lateral plasma membrane, we refer to CrhC as exhibiting polar-biased localization.

Polar-biased localization of a cold shock RNA helicase raises some intriguing implications for its role during cold acclimation. To survive cold shock, prokaryotic cells must acquire the ability to grow and divide under thermodynamically challenging conditions. A number of proteins required for these processes are plasma membrane associated. Thus, various aspects of bacterial sensing and response to cold shock as well as the recovery of cell growth and division capacity requires the *de novo* synthesis and insertion of these proteins into or across the plasma membrane. The most efficient mechanism to achieve this goal would involve the co-translational insertion of CSPs involving plasma membrane associated ribosomes. Similar to the polar and division plane localization of CrhC, ribosomes are concentrated at the cell poles and future division sites in *Bacillus subtilis* (Lewis *et al.*, 2000). In addition, ribosomes and CSPs also co-localize at the cell periphery in this organism (Mascarenhas *et al.*, 2001). These observations are not restricted to *Bacillus* as the major cold shock protein in *E. coli*, CspA, is polar

localized (Giangrossi *et al.*, 2001). Taken together with our localization data, these observations suggest ribosomes and CrhC may co-localize at the cell poles in cyanobacteria during cold shock.

CrhC may therefore function in a complex involved in the co-transcriptional translation and translocation (transertion) of cold shock-induced gene products into or across the plasma membrane. The 5′ to 3′ RNA helicase activity possessed by CrhC could potentially function in this complex to unwind secondary structure in the 5′ UTR of target mRNAs, allowing translation initiation to proceed. The resulting polypeptides would be directly inserted or transported across the plasma membrane. Potential transertion targets required for the acquisition and maintenance of a cold acclimated state are numerous. They include both the classical cold-induced lipid desaturase (Mustardy *et al.*, 1996; Los *et al.*, 1997) and periplasmic CSPs families (Barbaro *et al.*, 2002), as well as, a variety of proteins required for environmental sensing, cell division, and the cell cycle (Lybarger and Maddock, 2000; Aguilar *et al.*, 2001; Suzuki *et al.*, 2001; Kumar and Das, 2002). A significant number of these proteins are polar localized. The polar-biased localization of CrhC may therefore be a reflection of the requirement for transertion of these proteins into or across the plasma membrane at the cell poles. CrhC association with newly forming septa is also consistent with the transertion scenario, potentially providing proteins required for membrane, periplasm, or cell wall formation. A role for CrhC in cell division during cold shock cannot be ruled out. In *E. coli*, deletion of *csdA* resulted in an increase in doubling time and cell filamentation (Jones *et al.*, 1996), while disruption of an *Arabidopsis* RNA helicase gene initiates unregulated cell division in floral meristems (Jacobsen *et al.*, 1999). It is also interesting to note that the concept of polar is potentially

different in a functional context between single celled *E. coli* and *Bacillus* cells and filamentous *Anabaena*. In *Anabaena* the poles are located at the septa between adjacent cells and the transertion of proteins across the plasma membrane at this junction has the potential to function as a mechanism of cell-cell communication. Molecules involved in cell-cell communication include RNA, known as small RNA (Gottesman, 2004). Small RNAs have been described in the regulation of gene expression in response to environmental stresses including temperature (Johansson et al., 2002). Interestingly, the potential localization of the gold particles outside the cytoplasm may suggest that CrhC has been 'caught' during transport through a 'pore' or 'channel' of some sort between adjacent cells. In this scenario, CrhC may be involved in the transport of protein or RNA(s) at the poles and thus facilitating cell-cell communication and enhance the response for cold shock.

Although RNA helicase involvement in protein export has not been demonstrated, an RNA helicase was identified by a screen designed to identify exported or membrane-associated proteins in *Streptococcus pneumoniae* (Pearce *et al.*, 1993). The authors speculated that this helicase may be involved in the translation of polypeptides destined to be exported but further evidence has not been presented. A more detailed example of the potential for RNA helicase involvement in protein translocation involves SecA, an integral component of the Sec-dependent protein translocation system. Similar to CrhC, SecA possesses RNA helicase activity and is plasma membrane associated. Although the exact role that SecA driven RNA helicase activity performs in protein translocation is not known (Schmidt *et al.*, 2001; Sianidis *et al.*, 2001), it is possible that CrhC performs a function similar to that of SecA during cold acclimation in

cyanobacteria. In fact, evidence has been presented indicating that the Sec-dependent protein translocation process is cold sensitive (Pogliano and Beckwith, 1993).

Since RNA helicases are known to perform roles in all aspects of RNA metabolism, it is possible that CrhC is performing a function other than in translation initiation. The most likely candidate would be a role in RNA degradation. In *E. coli* the complex catalyzing RNA degradation, the RNA degradosome is plasma membrane associated (Liou *et al.*, 2001). The RNA helicase RhlB, a subunit in this complex, catalyzes dsRNA unwinding in the 3' to 5' direction providing ssRNA which is degraded by PNPase (Py *et al.*, 1996). In relation to a possible role for CrhC in RNA degradation during cold shock, Beran and Simons (2001) have provided evidence for the formation of a cold-adapted RNA degradosome in *E. coli*. The degradosome associated RNA helicase RhlB can be complemented *in vitro* by the *E. coli* RNA helicase CsdA, a CSP (Py *et al.*, 1996). This implies that CsdA is required for mRNA degradation during periods of cold shock. Indeed genetic evidence supporting the involvement of CsdA in the degradation of *E. coli* CSP mRNAs has been presented (Yamanaka and Inouye, 2001). In *Anabaena*, the plasma membrane localization of CrhC could be accounted for by its incorporation into a cold adapted degradosome, since degradosomes are plasma membrane associated in *E. coli* (Liou *et al.*, 2001). This would necessitate CrhC unwinding mRNA 3′ to 5′ providing ssRNA for degradation by PNPase. Although intriguing, a number of caveats to this theory exist. CrhC is neither the homologue of RhlB nor CsdA, and CrhC only unwinds artificial dsRNA unidirectionally 5′ to 3′ *in vitro* (Yu and Owttrim, 2000). Incorporation into a cold-adapted degradosome also does not readily account for the polar-biased and future division site localization of CrhC. This is especially true since a functional

degradosome is not required for normal growth of *E. coli* at low temperature (Beran and Simons, 2001). Finally, cyanobacteria and chloroplasts have been reported to lack the domain in RNase E required for degradosome assembly suggesting that degradosome-like complexes may not form in these systems (Baginsky, *et al.*, 2001).

Chapter 4

Identifying proteins co-purifying with the cyanobacterial cold shock-induced RNA helicase (CrhC) during cold shock

4.1 Introduction

Members of the DEAD box family of RNA helicases function in many cellular processes involving RNA metabolism, including transcription, RNA processing, export, stability and turnover, translation, and ribosome biogenesis (Tanner and Linder, 2001; Silverman *et al.*, 2003). RNA helicases contain seven conserved motifs required for ATP-binding, ATP hydrolysis, RNA binding and unwinding short stretches of duplex RNA in a progressive manner (helicase activity) (Linder and Stutz, 2001). To date, several members of the RNA helicase family have demonstrated RNA-dependent RNA helicase activity and/or RNA stimulated ATPase activity, while others are proposed to have RNA helicase (RNA unwinding) activity based on their sequence conservation with known helicases (Tanner and Linder, 2001). The fact that RNA helicases are involved in several essential cellular processes underlines a high specificity in their *in vivo* biochemical function (de la Cruz *et al.*, 1999). Many RNA helicases are known to function *in vivo* either in mulfi-subunit protein complexes, such as the eukaryotic translation initiation complex (eIF4A) (Rogers *et al.*, 2001), and the spliceosome (Stevens *et al.*, 2002) or in complex multi-molecular processes such as ribosome biogenesis (Nissan *et al.*, 2002), mRNA export (Dbp5) (Zhoa *et al.*, 2002), mRNA turnover (RhlB) (Py *et al.*, 1996), and protein secretion (SecA) (Schmidt *et al.*, 2001). Several approaches have been utilized to identify the function(s) of RNA helicases within these complexes including genetic, yeast 2-hybrid, co-purification, co-immunoprecipitation, GST pull-down, co-localization, and tandem affinity purification (TAP) (Silverman *et al.*, 2003; Butland *et al.*, 2005). The objective of the experiments presented in this section was to identify proteins interacting with CrhC using a combination of these techniques.

The cold shock-induced cyanobacterial RNA helicase, CrhC, possesses an ATP-independent RNA binding, (d)ATP-dependent RNA unwinding activity, and an RNA-dependent ATPase (Yu and Owttrim, 2000). Our initial approach to elucidate the function of CrhC involved cellular localization by membrane fractionation and immunogold labeling. CrhC was found to be plasma membrane associated with polar-biased localization at the septa separating two adjacent cells (Chapter 3; El-Fahmawi and Owttrim, 2003). Plasma membrane localization suggested that CrhC function(s) either in the regulation of RNA stability and decay as part of the cold-resistant degradosome or functions in a complex involved in the co-transcriptional translation and translocation (transertion) of cold shock-induced gene products into or across the plasma membrane (Chapter 3; El-Fahmawi and Owttrim, 2003). Previous work using native protein gel analysis and immunoprecipitation suggest the association of CrhC with a multi-subunit complex (Yu and Owttrim, 2000). Furthermore, [redacted]-Western analysis revealed a 37 kDa protein present in cold and normally grown *Anabaena* cell lysates which interacts with CrhC (Yu and Owttrim, 2000). The molecular weight of this protein is similar to that of ribosomal protein S1 from cyanobacteria and other related organisms. Also, the ATPase activity of CrhC was found to be efficiently stimulated by rRNA and polysome preparations. Thus, a potential role of CrhC in the cold shock response was proposed to be in translation initiation possibly through an interaction with Rps1 (Yu and Owttrim, 2000).

Here we report that the proteins co-purifying with CrhC are plasma and outer membrane associated, and CrhC co-localizes with ribosomes at the plasma membrane. These findings support the hypothesis that CrhC is involved in the transertion of proteins

into or across the plasma membrane during cyanobacterial acclimation to cold shock. A model for CrhC function during cold shock is proposed.

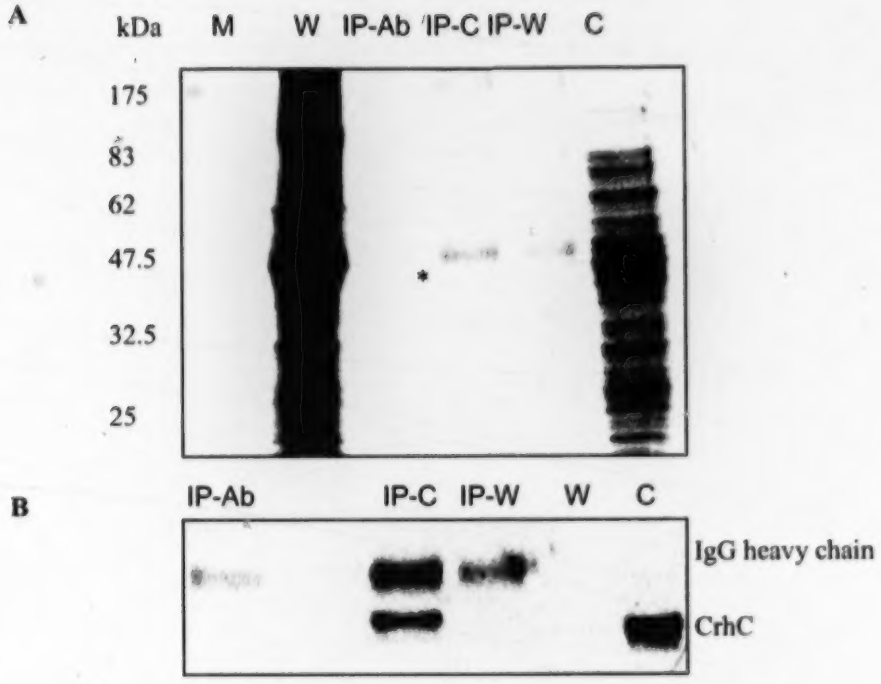
4.2 Results and discussion

Previously, CrhC was reported to be a component of a multi-subunit complex, although the identity of the interacting proteins was not determined (Yu and Owttrim 2000). Isolation and identification of specific protein(s) associated with CrhC by immunoprecipitation provides a better understanding of the role of CrhC during cold shock adaptation.

The protein fraction used for immunoprecipitation was obtained by solubilizing a crude membrane pellet with 1% sarcosyl as described in El-Fahmawi and Owttrim (2003). This fraction was diluted 10-fold to reduce the denaturing effect of sarcosyl as described in the materials and methods (Chapter 2, section 2.2.2.ii). The protein preparations from normal (30°C) and cold shocked cells (20°C) were subjected to immunoprecipitation by anti-CrhC antibodies (Fig. 4.1.A and B). Several polypeptides were detected by immunoprecipitation. However, these proteins correspond to the antibody as the protein banding pattern in the antibody (control), cold, and warm lanes were similar except for the protein band of 47 kDa in the IP-C lane (Fig. 4.1.A). The Western blot analysis of the immunoprecipitated proteins with anti-CrhC antibody indicates that the 47 kDa protein is CrhC (Fig. 4.1.B). The immunoprecipitation of CrhC is indicative of the high specificity of anti-CrhC antibody and the success of the employed IP procedure (Fig. 4.1.A and B, lane IP-C). The inability to detect co-immunoprecipitating proteins interacting with CrhC under these conditions is most

Fig. 4.1. Co-immunoprecipitation of proteins interacting with CrhC.

Polypeptide analysis of proteins co-immunoprecipitated with CrhC from *Anabaena* using anti-CrhC antibodies (Ab). Immunoprecipitation of 10-fold diluted membrane fraction prepared by solubilization of total protein extract from *Anabaena* cells either grown at 30°C (W) or 12 h cold shocked cells at 20°C (C) with 1% Sarcosyl. Immunoprecipitation of the protein extract was performed with anti-CrhC antibodies for cold (IP-C) and warm (IP-W). (A) Immunoprecipitated proteins were separated on a 10% SDS-PAGE gel and proteins were visualized by Coomassie blue staining. (B) Western blot analysis of immunoprecipitation products. The blot is probed with anti-CrhC antibody. (IP-Ab) Anti-CrhC coupled to Protein A-sepharose served as a control. (M) Molecular weight markers in kDa. The position of CrhC is indicated by an asterisk.


A
kDa
M
W
IP-Ab
IP-C
IP-W
C
175
83
62
47.5
32.5
25
B
IP-Ab
IP-C
IP-W
W
C
IgG heavy chain
CrhC

probably due to an irreversible denaturing effect of the anionic detergent, sarcosyl, on CrhC thereby breaking any protein-protein interaction(s).

4.2.1 *E. coli* proteins co-purifying with His_6-CrhC

To circumvent the need for detergent solubilization of CrhC, affinity chromatography was utilized to perform a His_6-CrhC pull-down experiment. Soluble lysates from cold shocked *Anabaena* were incubated with His_6-CrhC coupled to a Ni-NTA column which was then eluted with imidazole. SDS-PAGE analysis shows that a single polypeptide co-purified with His_6-CrhC from the affinity column (Fig. 4.2, His_6-CrhC+S1, one asterisk), which was not seen in the His_6-CrhC control or in the wash lane (Fig. 4.2). This protein was identified by mass spectrometry to be a degradation product of His_6-CrhC. These results triggered the speculation that His_6-CrhC from *E. coli* might be part of a multi-subunit protein complex, thus rendering it unavailable to interact with proteins from *Anabaena*. Indeed, analysis of the eluted fraction from the Ni-NTA resulted in the identification of 3 proteins that co-purify with CrhC (Fig. 4.3, lanes 40 and 250 mM imidazole). Despite extensive washing of the column with 20 mM imidazole, these proteins consistently eluted with His_6-CrhC. Furthermore, they were not observed in the wash fractions during the affinity purification but only in fractions containing CrhC (Fig. 4.3, Lanes W1, W2 and W3). The identified proteins are ribosomal protein S1 (Rps1), 2-oxoglutarate dehyrogenase decarboxylase, a component of the pyruvate dehydrogenase complex, and the outer membrane protein A (OmpA) (Fig. 4.3 and Table 4.1). In order to verify whether these proteins co-purify specifically with CrhC, the His_6-CrhC fraction was compared with the redox-regulated cyanobacterial RNA helicase (His_6-CrhR) fraction by SDS-PAGE. CrhR has biochemical characteristics similar to those exhibited

Fig. 4.2. Pull-down of proteins interacting with CrhC from *Anabaena*.

Analysis of proteins from 12 h cold shocked *Anabaena* cells that co-purify with His_6-CrhC. Total soluble protein extract from JM109 (pRSET29) in which CrhC was over-expressed was incubated with a Ni-NTA slurry. After binding for 1 h at 4°C, the slurry was divided into two columns. One column was eluted immediately with 40 mM imidazole to obtain His_6-CrhC to serve as a control (His_6-CrhC). The second column was incubated with the soluble protein extract (S) from *Anabaena* obtained by treating the pellet after cell disrubtion with 0.1 M of carbonate buffer pH 11 to obtain a mixture of peripheral membrane proteins and soluble proteins. FT is the flow through of the S fraction from the affinity column and His_6-CrhC + S fraction represents the proteins eluted with 40 mM imidazole, while (W) is the wash fraction of this column. The protein that co-purifies with $(His)_6$-CrhC indicated by an asterisk was identified by mass spectrometry.

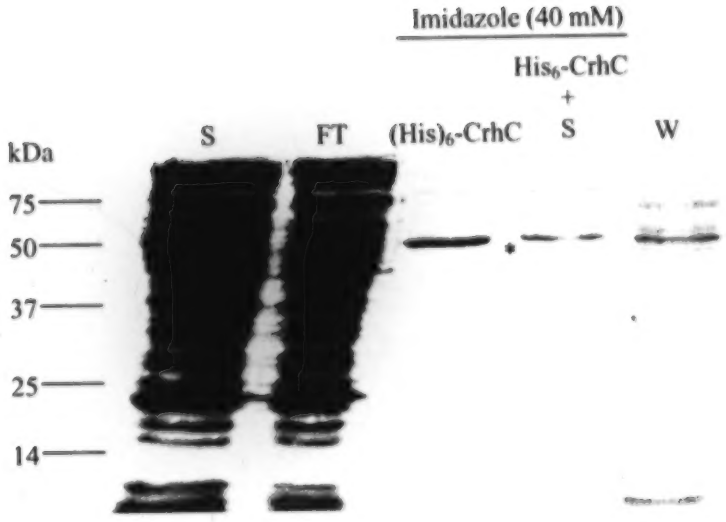

Imidazole (40 mM)
His6-CrhC
+
kDa
S
FT
(His)6-CrhC
S
W
75
50
37
25
14

Fig. 4.3. *E. coli* proteins co-purifying with CrhC.

Polypeptide analysis of affinity purified His_6-CrhC from JM109 (pRSET29). Lane (Sp) is the total soluble protein extract from JM109 pRSET where CrhC is over-expressed. Lane (FT) is the flow through of the Sp fraction after binding to the affinity column. W1, W2 and W3 are the washes of the affinity column with 10 mM imidazole buffer. Proteins eluted with 40 and 250 mM imidazole are shown in the two right hand lanes. Proteins that co-purify with His_6-CrhC are indicated by arrows and were identified by MALDI-TOF.

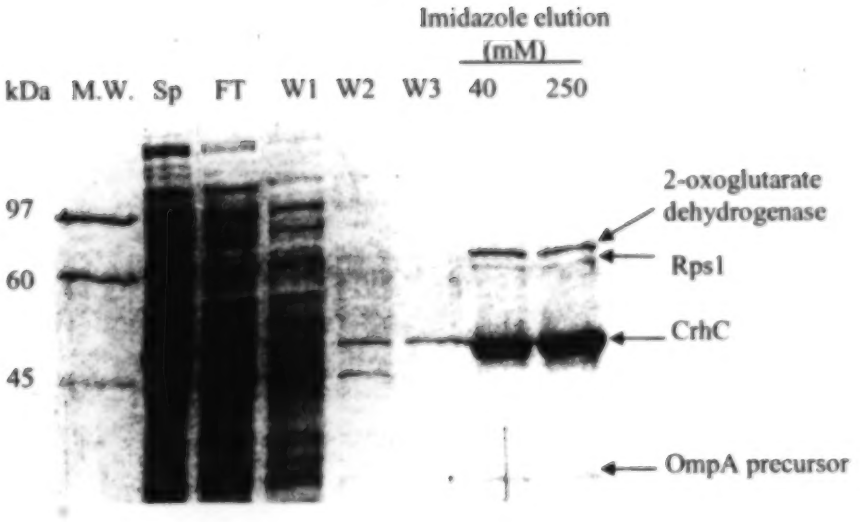

Imidazole elution
(mM)
kDa M.W. Sp FT W1 W2 W3 40 250
97
60
45
2-oxoglutarate
dehydrogenase
Rps1
CrhC
OmpA precursor

Table 4.1. Mass spectroscopy results of proteins co-purify with His_6-CrhC from the Ni-NTA affinity column. Results were analyzed by Mascot at http://www.matrixscience.com

Protein	No. of peptides[a]	MW (kDa)[b]	Function
2-oxogluterate dehydrogenase gi|43019	15	105	Catalyzes oxidative decarboxylation of pyruvate to acetyl coenzyme A (component of pyruvate dehydrogenase complex)
Ribosomal Protein S1 gi|223404	4	61	Translation initiation (component of 30S subunit)
Outer membrane protein A gi|129143	3	37.7	Major outer membrane porin protein

[a] The number represents the peptides identified by their masses generated by the mass spectrometer.
[b] The experimental molecular weight obtained by mass spectrometer analysis in kilo Daltons.

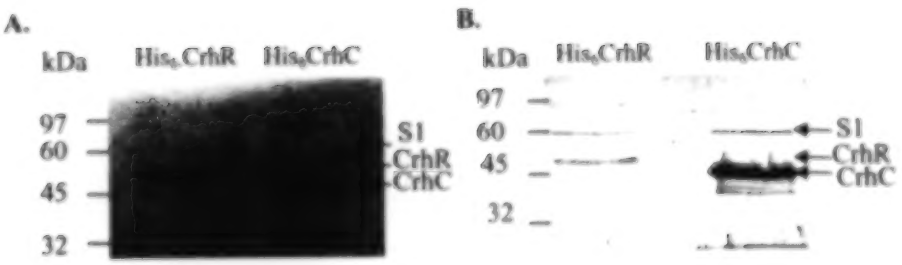
by CrhC, thus utilizing the same plasmid and strain of *E. coli* and under similar expression conditions served as a control. The eluted fractions from His_6-CrhC and His_6-CrhR were analyzed by SDS-PAGE (Fig. 4.4). Co-purifying proteins are not detected in the affinity purified fraction of His_6-CrhR as compared to His_6-CrhC (Fig. 4.4). In agreement with these findings, utilizing TAP several proteins have co-purified with DeaD/CsdA including CspA, CspC, CspD, cold-induced pyruvate dehydrogenase, Rps1, Rps2, Rps 3, Rps4, Hfq, OmpA (Butland *et al.*, 2005).

Two subunits of the *E. coli* pyruvate dehydrogenase complex are reported to be cold shock induced, dihydrolipoamide acetyl transferase and pyruvate dehydrogenase (Jones and Inouye, 1994). The third subunit of this complex is 2-oxoglutarate dehyrogenase decarboxylase. This membrane associated complex is essential to convert pyruvate acetyl CoA, which is an initial, important substrate for the tricarboxylic acid (TCA) cycle as well as for fatty acid biosynthesis (Yamanaka, 1999). This may suggest that the association of CrhC with 2-oxoglutarate dehyrogenase decarboxylase couples CrhC to the TCA cycle to sustain the ATP supply required for the ATP dependent active unwinding of thermodynamically stabilized secondary structures occurring at the 5′ UTR of mRNA.

In *E. coli*, Rps1 catalyzes an ATP-independent unwinding of secondary structures within the 5′ UTR of mRNA to a single stranded (ss) mRNA and the subsequent loading of the ssRNA on to the 30S small subunit of the ribosome. This is the initial step in translation initiation which is required for mRNA selection and recruitment (Tedin *et al.*, 1997; Sorensen *et al.*, 1998). However, during cold shock secondary RNA structure is thermodynamically stabilized and potentially requires active, ATP-dependent unwinding.

Fig. 4.4. Co-purification profile of CrhC and CrhR from *E. coli*.

Polypeptide analysis of affinity purified His_6-CrhC and His_6-CrhR. (A) Coomassie blue stained 10 % SDS-PAGE gel. (B) Western blot of purified His_6-CrhC and His_6-CrhR from the affinity column. The blot is probed with anti-CrhC and anti-Rps1 antibodies. Positions of His_6-CrhC, His_6-CrhR, and Rps1 are indicated by arrows. Asterisks mark *E. coli* proteins that co-purify with His_6-CrhC. Molecular weight markers are indicated in kDa.


A.
kDa
His6CrhR
His6CrhC
97
60
45
32
S1
CrhR
CrhC
B.
kDa
His6CrhR
His6CrhC
97
60
45
32
S1
CrhR
CrhC

Therefore, association of CrhC with Rps1 indicates that CrhC interacts with the 30S ribosomal subunit, and thus positions CrhC at the translation initiation step during cold shock. This association therefore supports the proposal that CrhC may perform active unwinding of the thermodynamically stabilized secondary structures present in the 5' UTR of cold shock-mRNAs, thereby, alleviating the block in translation initiation.

An example of secondary structures within the 5' UTR of mRNA is the *ompA* mRNA, which has two hairpin structures (Rosenbaum *et al.*, 1993; Rauhut and Klug, 1999). The translation of *ompA* mRNA may require ATP-dependent active unwinding by an RNA helicase to facilitate its translation during cold shock. However, in the case of *ompA* the requirement for an RNA helicase during translation initiation appears to be required not only for the destabilization of secondary structures but also for the displacement of RNA binding proteins which may act as translational repressors (Tanner and Linder, 2001). Recently, the translation repressor of *ompA* mRNA, host factor I (Hfq), was shown to bind *ompA* 5' UTR which increases at reduced growth rate (such as cold shock) resulting in the repression of its translation (Vytvytska *et al.*, 2000; Moll *et al.*, 2003). OmpA expression is constitutive and has not been reported to be cold-induced, and is required under all conditions to stabilize the outer membrane and maintain the rod shape of *E. coli* (Vytvytska *et al.* 2000; Moll *et al.*, 2003). Therefor the maintenance of active expression of OmpA is essential (Sonntag *et al.*, 1978; Vytvytska *et al.*, 2000; Moll *et al.*, 2003). Interestingly, the purified form of Hfq was reported to co-purify with Rps1 and SecA from *E. coli* (Sukhodolets and Garges, 2003; Butland *et al.*, 2005). SecA is an essential membrane associated component of the Sec-dependent protein system, and possess RNA helicase activity (Schmidt *et al.*, 2001; Sianidis *et al.*, 2001). Although, the

role performed by the SecA RNA helicase activity in protein translocation is yet to be elucidated, its association with Rps1, Hfq and OmpA may suggest that SecA is involved in translation by actively displacing Hfq, thus allowing the translation of *ompA* mRNA, and also the subsequent translocation of OmpA (Butland *et al.*, 2005). In fact, evidence has been presented indicating that the Sec-dependent protein translocation process is cold sensitive (Pogliano and Beckwith, 1993). Interestingly, in contrast to DeaD/CsdA, none of the CSPs has been shown to co-purify with SecA (Butland *et al.*, 2005). These findings may suggest that SecA function is complemented during cold shock by a cold -induced RNA helicase. A more intresting idea is that the co-purification of OmpA with CrhC may suggest a function of CrhC in transport through this porin for molecules involved in cell-cell communication by transporting a protein or RNA signaling molecules (Gottesman, 2004). In support of this idea, the expression of CrhC-GFP in *E. coli* displays a polar localization (data not shown) which is similar to its localization profile in *Anabaena* (El-Fahmawi and Owttrim, 2003).

It is well documented that RNA helicases can actively displace RNA binding proteins (e.g. Hfq), and to unwind in a progressive manner double stranded RNA utilizing energy from NTP hydrolysis (Linder *et al.*, 2001; Schwer, 2001; Rocak and Linder, 2004). It is possible that CrhC performs a function similar to that of SecA during cold acclimation as part of a cold adapted translocation complex. In this scenario, CrhC may function in displacing Hfq and destabilizing the secondary structures within the *ompA* 5′ UTR, allowing its translation initiation, and consequently transertion of proteins into and/or across the membrane.

4.2.2 Proteins co-purifying with CrhC from *Anabaena*

An alternative co-immunoprecipitation strategy was employed in which exogenous His_6-CrhC purified from *E. coli* was incubated with soluble proteins from warm and cold shocked *Anabaena* cells. His_6-CrhC was immunoprecipitated and co-purifying proteins, separated by SDS-PAGE, identified by mass spectrometry. However, the stripping of *E. coli* proteins co-purifying with His_6-CrhC during Ni-NTA affinity chromatograph was an essential initial step for this approach. Therefore, the soluble fraction from *E. coli* JM109 was treated with a non-denaturing detergent (octyl-D-glucoside) before affinity purification. This would gently disrupt the interaction of proteins associated with His_6-CrhC in *E. coli*, without denaturing CrhC. The purified His_6-CrhC fraction was analyzed by SDS-PAGE and Western blotting to determine if *E. coli* proteins were still associated with CrhC. The detergent treatment resulted in efficient stripping of proteins co-purifying with CrhC, where Coomassie staining shows no detectable proteins co-purifying with His_6-CrhC (Fig. 4.5., lane 2). Also, Western blot analysis with anti-Rps1 antibodies shows that the *E. coli* ribosomal protein S1 that normally co-purifies with His_6-CrhC (Fig. 4.6, lane 2), was not detected in the His_6-CrhC fraction after treatment with octyl-D-glucoside (Fig. 4.6, lane 3). The purity of His_6-CrhC was also tested by MALDI-MS, where only one protein was detected with a molecular weight of 51.5 kDa, the molecular weight of the His_6-CrhC (Fig. 4.7).

In *Anabaena*, CrhC solubilization by anionic detergents indicates tight association with the plasma membrane. The solubilization of CrhC is denaturing, results in the disruption of any possible CrhC-protein interactions, and thus made the

Fig. 4.5. Purification profile of His_6-CrhC from *E.coli* after detergent treatment.

Coomassie blue stained SDS-PAGE gel of His_6-CrhC affinity purified from *E. coli* after octyl-D-glucoside treatment. (1) Molecular weight markers. (2) His_6-CrhC purified from soluble fraction obtained from JM109 after treatment with the neutral non-denaturing detergent (1% octyl-D-glucoside).

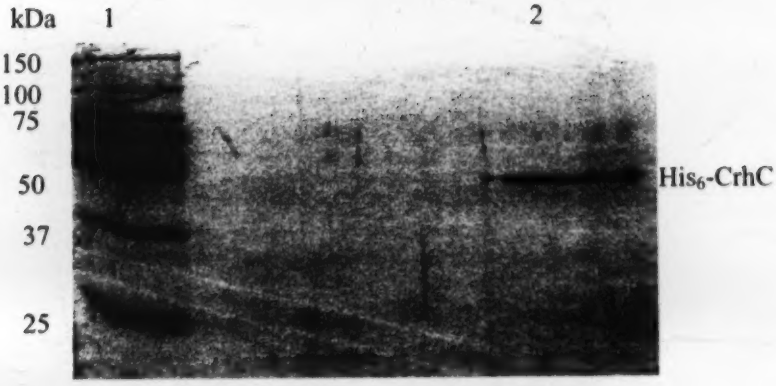

kDa
1
2
150
100
75
50
37
25
His6-CrhC

Fig. 4.6. His_6-CrhC purity examined by Western blotting.

Western blot analysis of the purity of the His_6-CrhC fraction obtained by affinity chromatography after treatment of the soluble extract with octyl-D-glucoside. Lane 1, Molecular weight markers. Lane 2, His_6-CrhC purified by affinity column chromatography from JM109 soluble fraction Lane 3, His_6-CrhC purified from soluble fraction obtained from JM109 after treatment with neutral non-denaturing detergent (1% octyl-D-glucoside). The blot is probed with (A) anti-CrhC antibodies and with (B) anti-Rps1 antibodies as indicated. The arrows indicate the positions of His_6-CrhC and ribosomal protein S1.

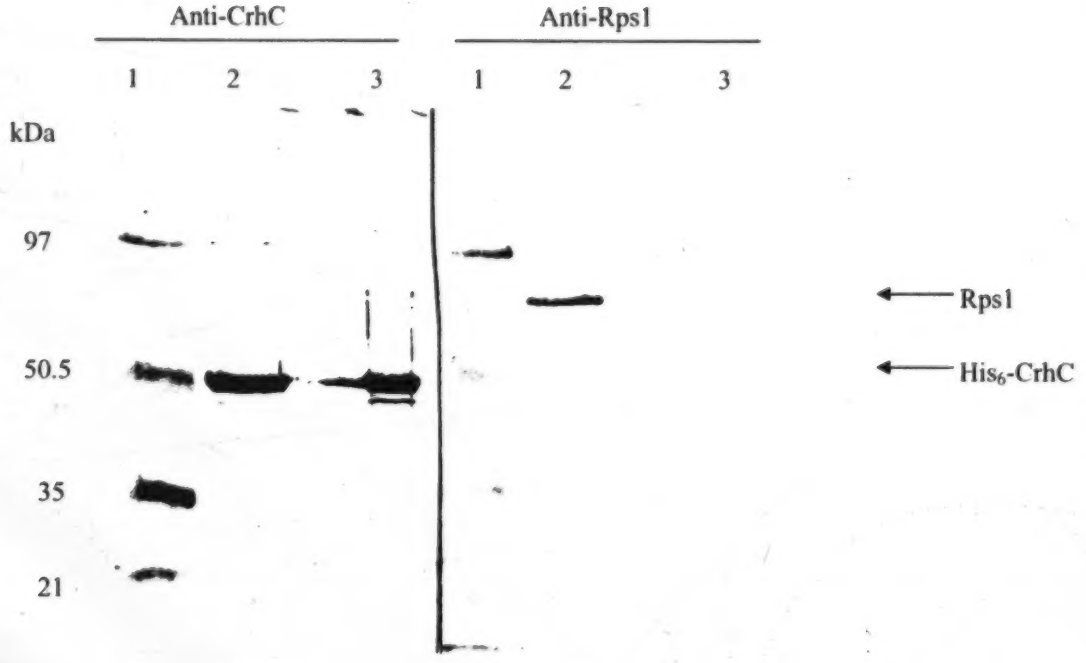
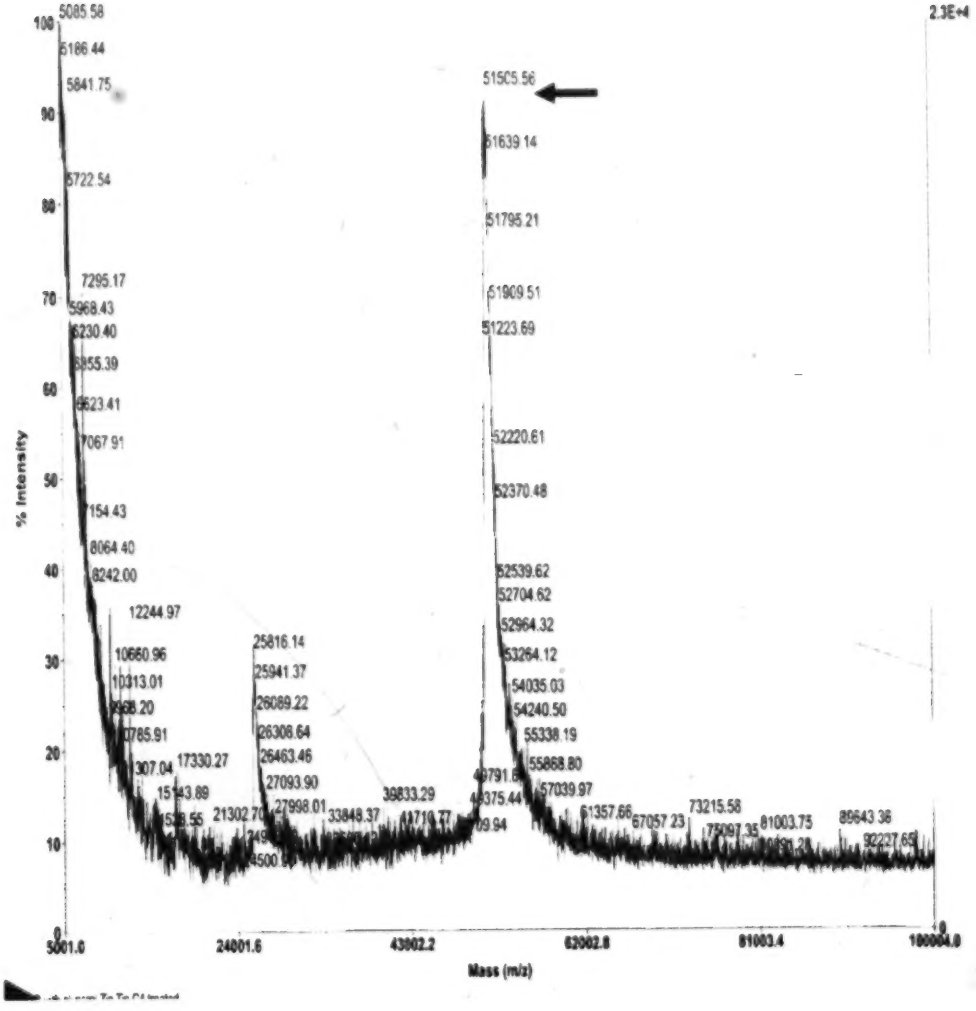

Anti-CrhC
Anti-Rps1
1
2
3
1
2
3
kDa
97
50.5
35
21
Rps1
His6-CrhC

Fig. 4.7. Mass spectrometer profile of affinity purified His_6-CrhC.

The highest peak represents the molecular weight of His_6-CrhC which is marked by the arrow.


Voyager Spec #1=>BC[BP = 5085.7, 23459]
% Intensity
Mass (m/z)
2.3E+4
5085.58
5186.44
5841.75
5722.54
7295.17
5968.43
6230.40
6355.39
6523.41
7067.91
7154.43
8064.40
8242.00
12244.97
10660.96
10313.01
9968.20
9785.91
9307.04
17330.27
15143.89
21302.70
25816.14
25941.37
26089.22
26308.64
26463.46
27093.90
27998.01
33848.37
39833.29
51505.56
51639.14
51795.21
51909.51
51223.69
52220.61
52370.48
52539.62
52704.62
52964.32
53264.12
54035.03
54240.50
55338.19
55868.80
57039.97
61357.66
67057.23
73215.58
81003.75
89643.38
5001.0
24801.6
43802.2
62002.8
81003.4
100004.0

immunoprecipitation of proteins associated with CrhC impossible (Section 4.2, Fig. 4.1). Therefore, His_6-CrhC stripped from co-purifying proteins was utilized in the co-immunoprecipitation experiment. Analysis of the co-immunoprecipitation products by Coomassie blue staining of a 10% SDS-PAGE gel identified 5 major proteins that co-immunoprecipitate with CrhC from cold shocked and normal grown cells (Fig. 4.8). The co-immunoprecipitated proteins were identified by MALDI-TOF-MS as 1) a 60 kDa hypothetical protein, 2) porin protein (major outer membrane protein), 3) ATP synthase subunit α, , 4) Nitrate transport protein, and 5) Apo cytochrome f (Table 4.2). Computer analysis with PSORT predicted the proteins to be membrane associated either to the plasma membrane or the outer membrane (Table 4.2). Despite the fact that mass spectrometry did not reveal a signal peptide in any of these proteins; it is most probable that these proteins are in their pre-translocation form, since the protein mixture utilized in the co-immunoprecipitation has originated from *Anabaena* treated with lysozyme, resulting in the digestion of the outer membrane.

The co-immunoprecipitation of these proteins with CrhC is intriguing. Based on the pull-down results from *E. coli*, the proteins that co-immunoprecipitate with CrhC were expected to be involved in translation or translocation of proteins across the plasma membrane. This was not the case as only membrane associated proteins were identified (Table 4.2). Two of the proteins that co-immunoprecipitated with CrhC are outer membrane protein homologues (Table 4.2). Blast analysis revealed that both proteins have an S-layer homology domain and a carbohydrate-selective porin domain, suggesting that a need for maintaining carbohydrate transport is required for cold shock tolerance. Interestingly, in *E. coli* the increased accumulation of trehalose [a non-reducing

Fig. 4.8. *Anabaena* proteins co-immunoprecipitating with His_6-CrhC.

SDS-PAGE analysis of *Anabaena* proteins that co-immunoprecipitate with His_6-CrhC. Lane 1, molecular weight markers. The soluble protein extract from *Anabaena* is a mixture of the cytosolic fraction and the supernatant fraction after incubating the pellet fraction with carbonate buffer wash (0.1 M, pH 11) to solublize peripheral membrane proteins from either (Lane 2) 12 h cold shocked cells at 20°C or (Lane 3) cells grown at 30°C. (Lane 4) protein fraction prepared from 12 h cold shocked cells immunoprecipitated with anti-CrhC antibodies; 5, protein fraction prepared from 12 h cold shocked cells; lane 6, from cells grown at 30°C, incubated with His_6-CrhC and then immunoprecipitated with anti-CrhC antibodies. Proteins were separated on a 10% SDS-PAGE gel and visualized by Coomassie blue staining. Proteins co-immunoprecipitating with His_6-CrhC were identified by MALDI-TOF-MS and are indicated by arrows. (A) Hypothetical 60 kDa protein 60. The protein band marked with (B) and (C) contained the 54 kDa porin protein (major outer membrane protein), and ATP synthase subunit α, 54 kDa. (D) CrhC, 51.5 kDa. (E) Nitrate transport protein (NtrA), 48.7 kDa. (F) Apo cytochrome f, 36 kDa. Details of the MALDI-TOF-MS results are provided in Table 4.2. The position of phycocyanin linker protein on the gel is indicated by an arrow.

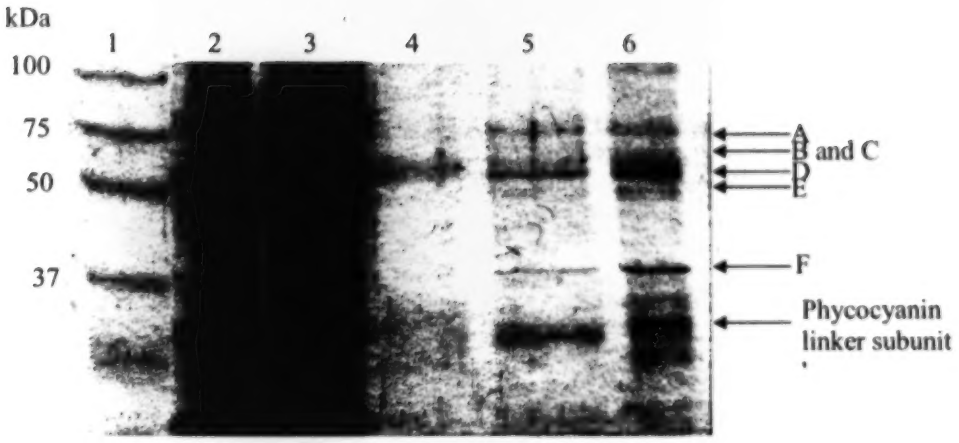

kDa
1
2
3
4
5
6
100
75
50
37
A
B and C
D
E
F
Phycocyanin
linker subunit

Table 4.2. Mass spectroscopy results of the proteins co-immunoprecipitated with His_6-CrhC from cold shocked and warm grown *Anabaena* cells. Results were analyzed by Mascot at http://www.matrixscience.com

Protein	No. of peptides[a]	MW (kDa)[b]	Cellular localization[c]
His-CrhC gi|17232042	15	51.5	Plasma membrane[d]
Hypothetical protein (S-layer homology domain and Carbohydrate-selective porin domain) gi|17232042	5	60.6	Outer membrane
Major outer membrane protein (S-layer homology domain and Carbohydrate-selective porin domain) gi|17228329	2	54.5	Outer membrane
ATP synthase α chain gi114508	1	54	Plasma membrane
Nitrate transport protein (NtrA) gi|10720398	3	48.7	Plasma membrane
Apocytochrome F gi|17229944	2	36	Plasma membrane

[a] The number represents the peptides identified by their masses generated by the mass spectrometer.
[b] The experimental molecular weight obtained by mass spectrometer analysis in kilo Daltons
[c]Cellular location of proteins were determined using PSORT at www.expasy.org.
[d]Cellular location published by El-Fahmawi and Owttrim, 2003.

disaccharide (α-D-glucopyranosyl-1,1-α-D-glucopyranoside)] is cold regulated (Kandror *et al.*, 2002). Trehalose is found at high concentrations in many organisms that naturally survive dehydration, for example, baker's yeast, some plants, and many bacteria (Crowe *et al.*, 1992). The ability of many of these organisms to survive cold shock also correlates with elevated trehalose content (Hottiger *et al.*, 1987; Van Laere, 1989; Crowe *et al.*, 1992). The addition of trehalose also provides protection against freezing, where exogenous trehalose enhanced the viability of bacteria (Israeli *et al.*, 1993) and yeast (Diniz-Mendez *et al.*, 1999) during freezing. Furthermore, although mammalian cells cannot produce trehalose, when trehalose-synthesizing enzymes from *E. coli* are expressed in human fibroblasts, these cells showed enhanced resistance to desiccation (Eroglu *et al.*, 1999; Guo *et al.*, 2000). The mechanism by which trehalose mediates tolerance to freezing or desiccation is not clear, but presumably involves a stabilization of certain cell proteins and/or membranes (Leslie *et al.*, 1994; Crowe *et al.*, 1998). It is noteworthy to mention that exogenous trehalose provides maximal protection against cold shock when it is present on both sides of the plasma membrane. This suggests that trehalose is required to stabilize cell membranes. Therefore, maintaining efficient transport of this sugar is required during temperature downshift (Lodato *et al.*, 1999; Kandror *et al.*, 2002). Thus, it is tempting to speculate that CrhC is required and/or essential for facilitating the translation and transertion of membrane proteins involved in maintaining carbohydrate transport (e.g. trehalose), cell shape, membrane integrity, and diffusion of proteins and substances across the membranes between adjacent cells or with the external environment.

As described previously, a decrease in membrane fluidity is one of the major physiological effects imposed by cold shock, which leads to a reduction of diffusion rates and the molecular dynamics of membrane function including membrane associated enzymatic networking, cell division, transport, protein translocation, and energy synthesis (Yamanaka, 1999; Phadtare *et al.*, 2000; Weber and Marahiel, 2003). Indeed, with respect to diffusion rate, it is interesting to note that *E. coli* outer membrane proteins OmpF (large porin) and OmpC (small porin) are differentially thermoregulated, where the expression of OmpF increases in response to cold shock, the expression of OmpC decreases (Lundrigan *et al.*, 1984; Nikaido and Vaara, 1985; Özkanca and Flint, 2002). The enhanced expression of OmpF suggests that there is selective expression for large porin proteins to counter balance the reduced diffusion rates imposed by cold. Interestingly, an antisense RNA gene, *micF*, is the major factor that thermoregulates *ompF* expression by an RNA/RNA interaction with the 5′ UTR (Andersen *et al.*, 1989; Delihas and Frost, 2001). The level of *ompF* mRNA in response to cold shock remains unchanged, however, the amount of OmpF protein increases, indicating that its expression is regulated at the level of translation (Delihas, 2003). In this scenario, an RNA helicase such as DeaD/CsdA from *E. coli* or CrhC from *Anabaena* might be required to destabilize the RNA/RNA interaction between the *ompF* and *micF* mRNAs thus promoting the translation of *ompF* mRNA during cold shock.

Another protein observed to co-immunoprecipitate with CrhC, the ATP synthase α subunit is an essential component of the membrane localized F_1F_o-type ATPases (Aggeler and Capaldi, 1996). F_1F_o-type ATPases utilize the energy of an H^+ electrochemical gradient (μ-H^+) generated by an H^+-pumping electron transport system to

generate ATP from ADP (Fillingame, 1997). A closely related F_1F_o-ATP synthase localizes to the thylakoid membrane in chloroplasts, where the enzyme functions in electron-transport-driven photophosphorylation (Fillingame, 1997).

Cytochrome F was also among the proteins that co-immunoprecipitated with CrhC. Cytochrome F is a thylakoid membrane protein involved in photosynthetic electron transport. Cytochrome F has an internal chain of five water molecules that has been proposed to form a "proton wire" and act as the exit-port for proton translocation generated through photosynthesis. The resulting proton gradient is used by the ATP synthase to form ATP (Martínez *et al.*, 1996). Initially, it seems surprising that the precursor polypeptide of the thylakoid membrane protein, cytochrome F, co-immunoprecipitates with CrhC, however, this may suggest that CrhC is not only playing a role in the transertion of plasma membrane proteins, but also for thylakoid membrane proteins. This is a plausible scenario as the plasma membrane and thylakoid membrane are contagious, where membrane protein exchange may occur between the plasma and the thylakoid membrane (Peschek *et al.*, 1989).

The fifth plasma membrane associated protein co-immunoprecipitating with CrhC, was nitrate transport protein (NtrA). NtrA is essential for maintaining nitrate transport from the medium into the cells when nitrate becomes limiting (Jose´ *et al.*, 1997). The repression of heterocyst initiation in an RNA binding protein gene mutant (*rbpA1*$^-$) in *Anabaena* M3 strain, has suggested a functional link between cold shock and global nitrogen metabolism in cyanobacteria (Sato and Wada, 1996). Furthermore, Sakamoto and Bryant (1997) have shown that cold shock in *Synechococcus* sp. strain PCC 7002 results in nitrogen limitation when grown in a medium containing nitrate as

the sole nitrogen source. Recently, the nitrogen status was reported to modulate the expression of RNA binding proteins genes, Rbps, in cyanobacteria during cold shock (Mori *et al*., 2003). Based on the above, maintaining NtrA synthesis during cold shock is essential in order overcome nitrate limitation caused by cold shock. Also, maintaining nitrogen transport will ensure the regulation of cold shock genes expression such as *rbpA*, *rbpB*, *rbpC*, and *rbpD*.

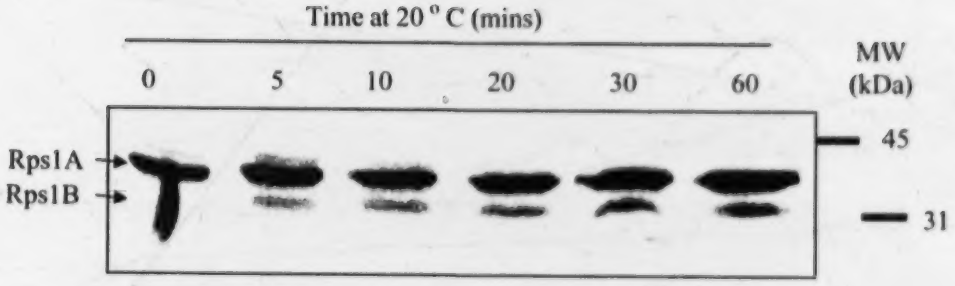
The co-immunoprecipitation protein profile of CrhC included proteins that are targeted to both the plasma and outer membrane, which suggests that CrhC may have a role in protein translocation into and cross the plasma membrane during cold shock.

4.2.3 *Anabaena* Rps1 expression in response to cold shock

Western blot analysis with anti-Rps1 antibody of subcellular fractions from cold shocked and normal grown *Anabaena* cells revealed a difference in the expression profile of Rps1 (Fig 3.2). Further analysis of Rps1 expression in *Anabaena* was therefore warranted. Blast searches of the *Anabaena* genome sequence using the *E. coli* Rps1 sequence revealed that the *Anabaena* genome encodes two ribosomal protein S1 genes, the 37 kDa, Rps1A, and the 33 kDa, Rps1B. To determine the potential role of Rps1 in the cold shock response in *Anabaena*, Western blot analysis was performed. Anti-Rps1 antibody against both *E. coli* and *Synechosystis* was generously provided by Dr. P. Baumann (University of California). Western analysis indicates that the Rps1A protein is constitutively expressed in response to temperature change, while Rps1B is temperature regulated (Fig. 4.9). Rps1B cold induction is rapid, being detected within the first 5 mins of cold shock and its concentration increases as the cold shock progresses (Fig. 4.9). This

Fig. 4.9. The expression of Rps1 in *Anabaena* during cold shock

Western blot analysis of the two isoforms of ribosomal protein S1 from *Anabaena* during exposure to cold shock at 20°C for the indicated times. The blot was probed with anti-Rps1 antibody. The position of Rps1A and Rps1B is indicated by arrows.


Time at 20 ° C (mins)
0
5
10
20
30
60
MW
(kDa)
Rps1A
Rps1B
45
31

rapid cold induced expression of Rps1B may be required for instantaneous modification of the ribosome to become cold adapted. Furthermore, such modification of the ribosome may not only contribute to a cold adapted ribosome but may also help in prioritizing the CSPs mRNA in loading onto the 30S subunit for translation to be initiated.

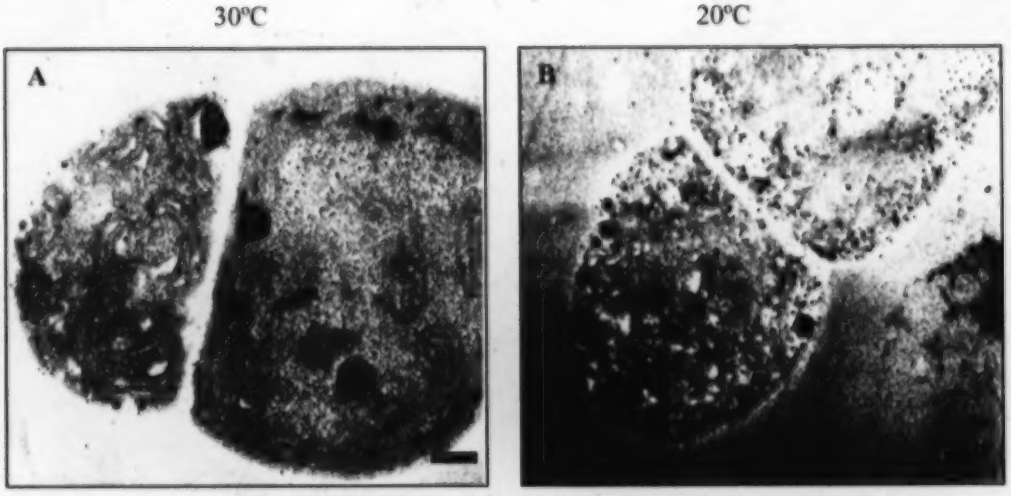
The evidence for CrhC association with the ribosome was initially reported by Yu and Owttrim, (2000), where CrhC was found to interact with a ≈37 kDa protein from both cold and warm cells and hypothesized to be the ribosomal protein S1. Also, an association of CrhC with Rps1 was seen in *E. coli*, where Rps1 from *E. coli* co-purified with His_6-CrhC suggesting an association of CrhC with the ribosome, which could by achieved by an interaction with Rps1. Despite the fact that Rps1 was not among the identified co-immunoprecipitated proteins with His_6-CrhC from normal and cold shocked cells from *Anabaena*, the possibility that CrhC may interact and/or associate with the ribosome transiently during cold shock cannot be excluded.

4.2.4 CrhC co-localizes with the ribosome

Immunochemical localization of the ribosome in normal and cold shocked cells was achieved utilizing anti-Rps1 antibodies. Interestingly, in *Anabaena*, in response to cold shock the ribosomes predominantly localize around the nucleoid, extending to close proximity to the plasma membrane (Fig. 4.10.B), which is significantly different from their distribution pattern during normal growth temperature (Fig. 4.10.A). Moreover, the relative localization of CrhC and the ribosome at the plasma membrane was determined utilizing anti-CrhC and anti-Rps1 antibodies.

The double labeling suggests that a proportion of ribosomes (5 nm gold particles) co-localize with CrhC (10 nm gold particles) at the plasma membrane, mostly at the poles

Fig. 4.10. IEM subcellular localization of ribosomes in *Anabaena* after cold shock. Ribosome distribution within an ultrathin section of an *Anabaena* filament using anti-Rps1 polyclonal antiserum at 1:100 dilution, (A) grown at 30°C and (B) cells cold shocked for 6 h at 20°C. The gold particles were 5 nm in size, indicate the distribution of ribosomes in *Anabaena.* Bar = 500 nm


30°C
20°C
A
B

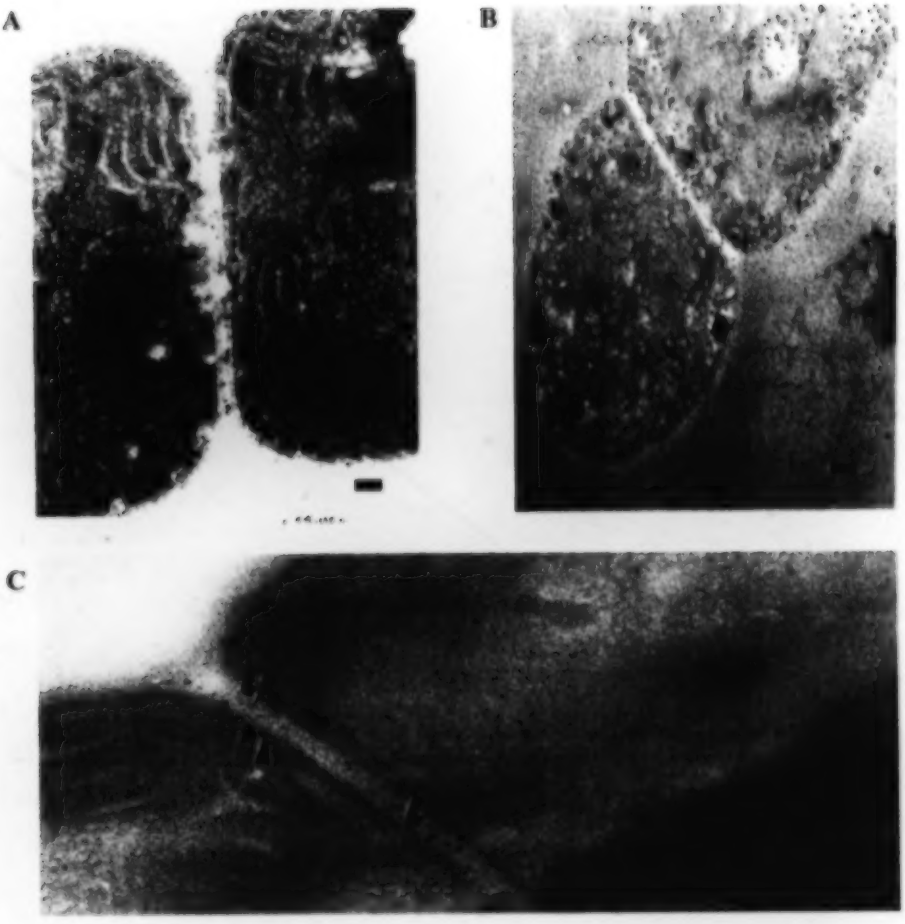
(Fig. 4.11.C). In support of these results, the co-purification of several components of the 30S subunit including Rps1 and Rps2 strongly suggests the association of this RNA helicase with translation initiation (Butland *et al.*, 2005). Futhermore, *in vivo* localization studies of GFP-CspB revealed that CSPs co-localize with ribosomes and this localization is polar, and depends on active transcription in *B. subtilis* (Weber *et al.*, 2001; Mascarenhas *et al.*, 2001). A similar observation was reported for the major cold shock protein in *E. coli*, CspA, which is also polar localized (Giangrossi *et al.*, 2001). Also, in *Streptomyces aureofaciens*, CSPs co-purify with ribosomes (Mikulik *et al.*, 1999).

Polysomes translating pre-secretory proteins associate with membranes, where it is proposed that while mRNA is being translated the emerging polypeptide from the ribosome is threaded through the dynamic protein channel, the translocase (Keyzer *et al.*, 2003). Bacteria contain two major protein targeting routes, the Sec and signal recognition particle (SRP) mediated pathways, which both utilize the general secretion pathway for protein translocation and targeting into and cross the membrane via the translocase (for a recent review see, Keyzer *et al.*, 2003). Protein translocation can occur either post-translationally (Sec route) or co-translationally (SRP route). The common component between the two pathways is SecA (Facey and Kuhn, 2004; Vrontou and Economou, 2004; Karamanou *et al.*, 2005). In response to cold shock, wild type *E. coli* and *B. subtilis* display reduced efficiency in the protein translocation compared to optimal growth temperatures, and thus it was concluded that the general secretion pathway is cold sensitive (Pglianom and Beckwith, 1993; Bolhuis *et al.*, 1998).

In both protein targeting routes, the trigger factor (TF), (cis-trans proline isomerase), which associates with the 50S ribosomal subunit, scans the newly

Fig. 4.11. IEM of the co-localization of CrhC with the ribosome.

ImmunoGold labeling of ultrathin sections of 6 hr cold shocked *Anabaena* cells at 20°C with anti-CrhC, and anti-Rps1. (A) Cells probed with anti-CrhC, 10 nm gold particles indicate CrhC. (B) Cells probed with anti-S1, 5 nm particles indicate ribosome distribution. (C) Co-localization of CrhC with ribosomes, the wide arrowheads point to CrhC, and narrow head arrows points to ribosomes. Bar = 500 nm


A
B
C

synthesized proteins while bound to the ribosome, and determines if the nascent polypeptide is to be SRP or Sec targeted (Hesterkamp *et al.*, 1996; Lee and Bernstein, 2002). TF expression is cold-induced, and enhances the viability of *E. coli* (Kandror and Goldberg, 1997). Moreover, it has been reported that *lspA*, the gene encoding the signal peptidase II in *B. subtilis*, is required for the efficient secretion of α amylase and is essential for growth at 15ºC (Tjalsma *et al.*, 1999). These data suggest that bacteria upregulate the expression of protein secretion, and the maintenance of protein export is indicative of the importance of this function during cold shock.

Furthermore, an essential component of the general secretion pathway is SecA which functions as an ATP dependent motor that drives the protein translocation process at the translocase (Driessen, 1993; Akita *et al.*, 1991). Moreover, SecA also possess an RNA helicase activity, and both are required for protein translocation (Koonin and Gorbalenya, 1992; Schmidt *et al.*, 2001; Sianidis *et al.*, 2001; Baud *et al.*, 2002). The requirement for an RNA helicase in protein targeting and translocation seems logical, where mRNA sequences of type III secreted proteins was hypothesized to contain their translocation signal (Anderson and Schneewind, 1997 and 1999; Anderson *et al.*, 1999; Anderson *et al.*, 2002). A possible role for an RNA helicase such as CrhC in this scenario might be to expose this signal sequence to be recognized for co-translation translocation processes into and across the plasma membrane.

4.3. CrhC and the cold shock response: A model.

Therefore, taken together with CrhC localization data (El-Fahmawi and Owttrim, 2003), the co-localization observations of ribosomes with CrhC at the plasma membrane,

and the co-immunoprecipitation results, a model is proposed for CrhC function during cold shock. The major metabolic block during cold shock is proposed to occur at the level of translation initiation. However, a set of CSPs are selectively synthesized in response to cold shock, which suggests that the ribosome becomes cold adapted and has the ability to selectively translate cold shock mRNAs. In support of this idea, many of the cold shock upregulated proteins in *E. coli* are associated with the translation apparatus, such as initiation factors (IF1, IF2, and IF3), RbfA, CsdA, and TF (Gualerzi *et al*., 2003; Weber and Marahiel, 2003; Inouye and Phadtare, 2004; Giuliodori *et al*., 2004). Similarly, in cyanobacteria, the cold up regulated Rps1B, ribosomal protein S21 and Rpbs may contribute to the formation of a cold adapted ribosome.

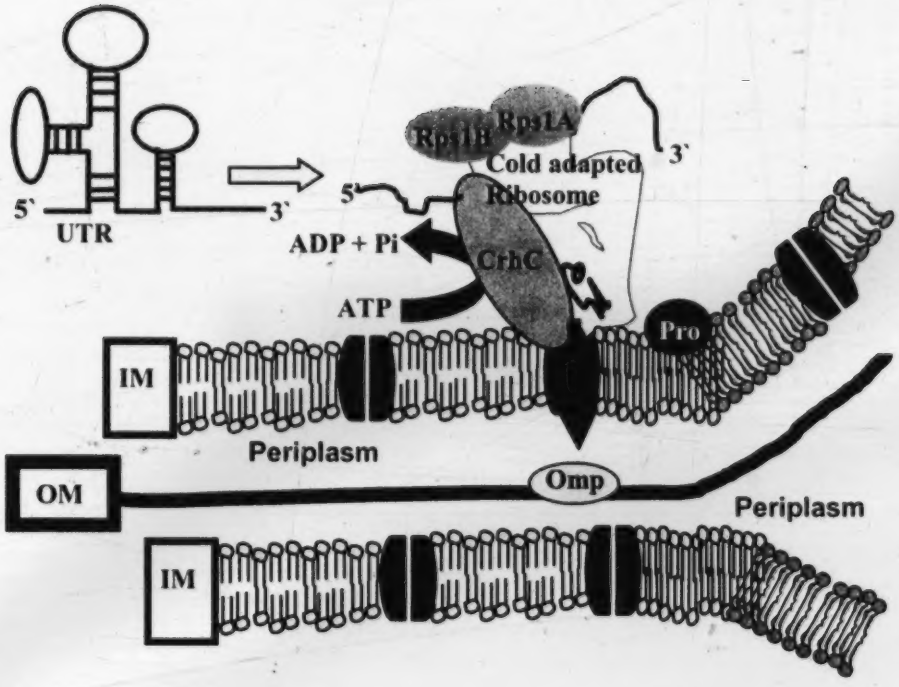
The two primary interactions between mRNA and 30S subunits at the translation initiation step, Rps1-mRNA binding and the Shine-Dalgarno mRNA-rRNA interaction, both require locally single stranded mRNA (Schlax and Worhunsky, 2003). For translation to be initiated the 5′ UTR has to be cleared of secondary structure and/or RNA binding proteins that may cause translation repression (Schlax and Worhunsky, 2003). During cold shock secondary structure within the 5′ UTR become thermodynamically stable and thus imposes a major obstacle for translation initiation. The ability of CrhC to unwind secondary structure occurring in the 5′ UTR, and its ability to displace RNA binding proteins that may repress translation such as Hfq, protein Y and Rbps positions CrhC at the translation initiation step with Rps1. However, the co-immunoprecipitation profile of CrhC displays a mixture of plasma and outer membrane proteins suggesting that CrhC may function as part of a cold adapted translocation apparatus similar to Sec A which may target proteins into or across the plasma membrane. Proteomic analysis of the

periplasmic proteins from *Synechocystis* in response cold and osmotic shock, showed that 47 of the 57 proteins identified had a typical Sec-dependent signal peptide, suggesting that this translocation apparatus is cold adapted (Fulda *et al.*, 2000).

In this capacity, CrhC is proposed to function in a complex involved in the co-transcription, and translation of CS mRNA on cold adapted ribosomes which are membrane associated. The nascent polypeptides of cold shock-induced gene products can then be directly translocated into or across the plasma membrane, thereby maintaining normal cell shape, membrane integrity, nitrogen metabolism, electron transport, and diffusion of substances across the membranes between adjacent cells or with the external environment during cold shock (Fig. 4.12).

Fig. 4.12. A model for CrhC role during cold shock.

A schematic diagram of the membranes at the septa separating two adjacent *Anabaena* (poles), illustrating the proposed function for CrhC during cold shock (see text for details).


5`
UTR
3`
Rps1B
Rps1A
3`
Cold adapted
Ribosome
5`
ADP + Pi
CrhC
ATP
Pro
IM
Periplasm
OM
Omp
Periplasm
IM

Chapter 5

Cold enhanced phosphorylation of EF-TU and Rps2 in the cyanobacterium *Anabaena* sp. strain PCC 7120.

This chapter has been submitted to the Journal of Microbiology.

5.1 Introduction

Free-living prokaryotic organisms are capable of responding to a variety of environmental stresses, including fluctuations in temperature. Cold shock is a common stress encountered by prokaryotes in nature, affecting a diverse range of physiological parameters in living cells. Specifically, growth at reduced temperature most significantly reduces both the efficiency of protein synthesis and membrane fluidity (reviewed in Jones and Inouye, 1997, Ermolenko and Makhatadze, 2002, Weber and Marahiel, 2003, Gualerzi *et al.*, 2003, and Phadtare, 2004). A long standing problem, and an apparent contradiction of growth at low temperature, is the inhibition of global protein synthesis while expression of a specific set of genes, encoding the cold shock proteins (CSPs), is induced. A small but growing list of proteins comprise the CSP family, including the CspA (Goldstein *et al.*, 1990), CspB (Lee *et al.*, 1994), CspG (Nakashima *et al.*, 1996), CspI (Wang *et al.*, 1999), Rbp family of RNA binding proteins (Sato, 1995), RNA helicases (Jones *et al.*, 1996; Chamot *et al.*, 1999; Lim *et al.*, 2000; Chamot and Owttrim, 2000), and fatty acid desaturases (Los *et al.*, 1997), H-NS (La Teana *et al.*, 1991), NusA (Friedman *et al.*, 1984), PNPase (Donovan and Kushner, 1986), RecA (Walker, 1984), IF2 (Gualerzi and Pon, 1990), IF1, IF3 (Giuliodori *et al.*, 2004), H-NS (Dersch *et al.*, 1994), the α subunit of DNA gyrase (Sugino *et al.*, 1977), trigger factor (TF) (Kandror and Goldberg, 1997). CSPs can therefore generally be assigned a role in the maintenance of nucleic acid or plasma membrane integrity or function. CSP expression is regulated at the post-transcriptional level, through a combination of enhanced RNA stability and preferential translation of CSP mRNA (Chamot and Owttrim, 2000; Giuliodori *et al.*, 2004; Thieringer *et al.*, 1998; Yamanaka, 1999: Golovlev, 2003; Gualerzi *et al.*, 2003;

Weber and Marahiel, 2003; Phadtare, 2004). However, the mechanism(s) by which bacteria selectively inhibit general mRNA translation and enhance CSP mRNA translation during periods of growth at low temperature remain ill defined.

Ribosome involvement in the cold shock response was first described by Das and Goldstein (1968) and Friedman *et al.*, (1969) who observed that an unknown component(s) of the bacterial ribosomal fraction is responsible for the arrest of translation at low temperature. van Bogelen and Niedhardt (1990) extended this concept by proposing that the ribosome functions as the bacterial thermosensor, suggesting that the state of the ribosome signals the induction of temperature responses. From these and other observations, it has become clear that a major effect of cold shock is the formation of a cold adapted ribosome, through association with a variety of protein factors. A number of these factors are proposed to function in translation initiation including, translation initiation factors IF2 (Jones *et al.*, 1987), IF1, and IF3 (Guarlezi *et al.*, 2004). Many are synthesized *de novo* in response to cold stress, including Ribosome binding protein factor A (RbfA) (Jones and Inouye, 1996) and cold shock DEAD RNA helicase (DeaD/CsdA) (Jones *et al.*, 1996). Although not directly required for translation initiation, protein Y has recently been shown to associate with 70S ribosomes by binding the 30S ribosomal subunit in *E. coli* (Vila-Sanjurjo *et al.*, 2004). Protein Y binding blocks both the peptidyl- and aminoacyl-tRNA sites thereby inhibiting ribosome dissociation and potentially translation initiation during cold stress.

Translation can be divided into three main steps; initiation, elongation, and termination, each of which have been shown to be affected by temperature downshift. Initiation requires interaction between the IF factors and Rps2 and Rps1 which load

mRNA onto the 30S ribosomal subunit, to form a translation initiation complex. The efficiency of this reaction is affected by secondary structure at the 5′ end of the mRNA whose stability will be enhanced at reduced temperature (Pelletier and Sonenberg, 1985). Temperature effects on translation elongation have also been proposed to be involved in low temperature regulation of translation in *E. coli*. In 1996, Graumann and Marahiel proposed that the efficiency of translation is dependent upon the temperature-dependent occupancy of the ribosomal A-site by aminoacyl-tRNA during translation elongation. The first step in translation elongation involves aminoacyl-tRNA binding to the ribosomal A-site in a codon-dependent manner. This reaction requires elongation factor Tu (EF-Tu) and GTP, which form a ternary complex with aminoacyl-tRNA. Phosphorylation of EF-Tu on the conserved residue, threonine-382 (Lippmann *et al.*, 1993; Chang and Traugh, 1997), prevents ternary complex formation, thereby resulting in an inhibition of translation elongation (Lippmann *et al.*, 1993; Alexander *et al.*, 1995). The phosphorylation state of EF-Tu is therefore a crucial factor regulating the rate of translation.

In this study we investigated the pattern of cold-induced protein phosphorylation in the cyanobacterium, *Anabaena* sp. strain PCC 7120, *in vitro* and *in vivo*, for the purpose of identifying downstream targets in the cold response signal transduction pathway. Two phosphoproteins were purified and identified, translation elongation factor (EF-Tu) and ribosomal protein S2 (Rps2), which are rapidly and transiently hyperphosphorylated in response to cold stress. In addition, mass spectrometry analysis suggests that the two proteins are further modified by methylation and/or acetylation during cold stress. Phosphorylation of Rps2 and EF-Tu are proposed to initiate a decrease

in cellular capacity to perform translation initiation and elongation, respectively. The results indicate that an immediate response of the cyanobacterial cell to cold stress involves downregulation of both translation initiation and elongation through post-translational modification of ribosome-associated proteins. The results therefore provide insight into the mechanism by which low temperature leads to the rapid reduction in general protein translation.

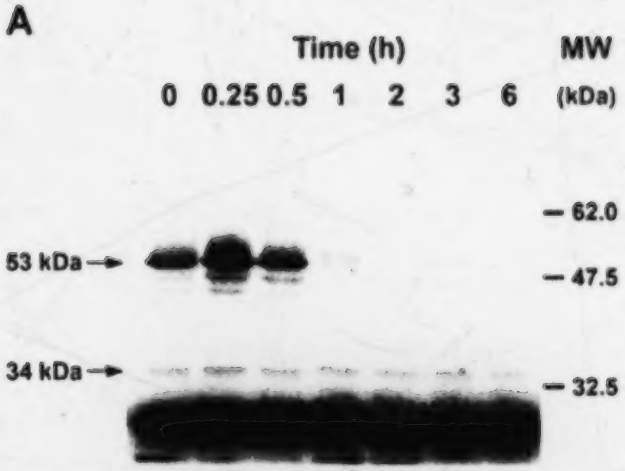
5.2 Results

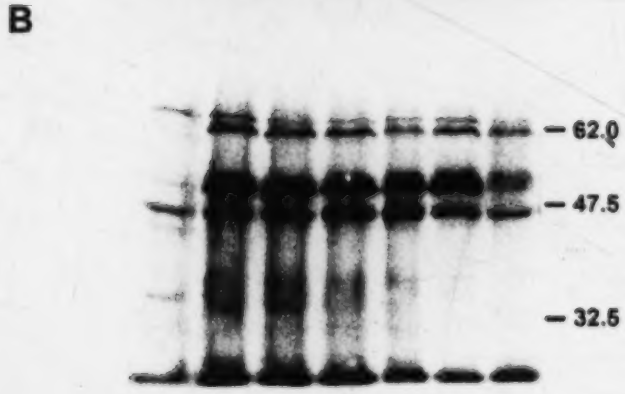
5.2.1 Effect of cold stress on the *in vitro* phosphorylation profile of *Anabaena* proteins.

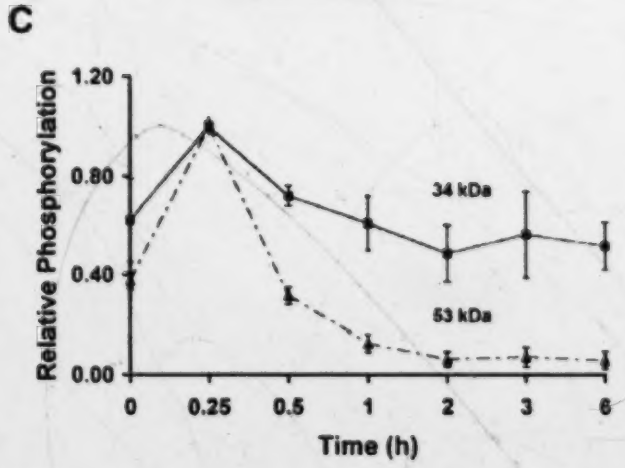
phosphorylation was used to investigate the phosphorylation profile of cold stress proteins in order to identify component(s) of the cold shock response signal transduction pathway. Phosphorylation of a number of proteins was observed during a time course of cold stress (Fig. 1A). To confirm the low temperature specificity of the observed phosphorylation pattern, the same profile was examined in response to salt stress (Fig. 1B). Comparison of the two figures indicates that each stress induces a unique phosphorylation profile. Although the phosphorylation of several proteins changed in response to cold stress, quantification revealed two predominant proteins that displayed a consistent increase in their relative phosphorylation within 0.25 h of temperature downshift (Fig. 1C). While both proteins are phosphorylated at all time points, phosphorylation levels within 0.25 h of cold shock of the 53 kDa and 34 kDa proteins increased 2.6- and 1.6-fold when compared to their phosphorylation level at zero time respectively, in response to cold shock. Phosphorylation levels of both proteins decreased

Fig 5.1. *In vitro* phosphorylation profile of the cold shock response in *Anabaena*.

In vitro phosphorylation profile of *Anabaena* protein extracts from a 6 h time course of cold shock (A) or salt stress (B). The hyperphosphorylated proteins are indicated with arrows (53 kDa, 34 kDa). (C) Relative phosphorylation of the 53 kDa and 34 kDa proteins in response to cold shock. The results are a representation of three independent experiments, a typical representation of which is shown in (A). The quantification of phosphorylated bands was performed utilizing ImageQuant v.1.2, assuming the value at time 0.25 has a value of 1.


A
Time (h)
MW
0 0.25 0.5 1 2 3 6
(kDa)
53 kDa
34 kDa
62.0
47.5
32.5


B
62.0
47.5
32.5


C
Relative Phosphorylation
1.20
0.80
0.40
0.00
34 kDa
53 kDa
0
0.25
0.5
1
2
3
6
Time (h)

at 0.5 h cold shock to a level comparable to the zero time phosphorylation level, which is indicative that both proteins are specifically subjected to hyperphosphorylation within 0.25 h of cold shock with subsequent dephosphrylation to background and thus they are transiently hyperphosphorylated (Fig. 1C).

5.2.2 The 53 kDa protein is phosphorylated on a threonine residue.

Western blot analysis was used to identify which amino acids were phosphorylated. Monoclonal and polyclonal (data not shown) anti-phosphothreonine antisera both detected a 53 kDa polypeptide in cell extracts isolated from cells subjected to cold stress (arrow, Fig 5.2A). The intensity of the signal was maximal at 0.25 h and decreased with increasing time, in a pattern similar to the *in vitro* phosphorylation profile (compare Fig. 5.1A and Fig. 5.2A). Western analysis of the same extracts with anti-phosphotyrosine or anti-phosphoserine antisera, did not detect any cross-reaction (data not shown). The results indicate that a 53 kDa polypeptide is phosphorylated on a threonine residue in response to cold stress. Furthermore, anti-phosphothreonine immunodecoration revealed a different pattern of accumulation of the 53 kDa polypeptide from salt stressed extracts (compare Fig. 5.2A and Fig. 5.2B), suggesting that the threonine phosphorylation of the 53 kDa protein is cold stress specific.

5.2.3 Purification of cold stress-induced phosphorylated proteins from *Anabaena*.

Phosphorylated proteins present in cold stressed *Anabaena* were purified using Immobilized Metal Affinity Chromatography (IMAC) with a phosphoprotein purification kit (QIAGEN). This approach permits the purification of proteins phosphorylated on serine, threonine, or tyrosine residues, allowing phosphoprotein identification by mass spectrometry. Two polypeptides with molecular masses of 53 kDa (arrow) and 34 kDa

Fig. 5.2. Phosphorylation profile of the cold shock response in *Anabaena*.

Western blot analysis of cell free protein extracts from a 6 h time course of (A) cold and (B) high salt stressed cells from *Anabaena* at the indicated time points. The blot was probed with monoclonal anti-phosphothreonine antiserum.

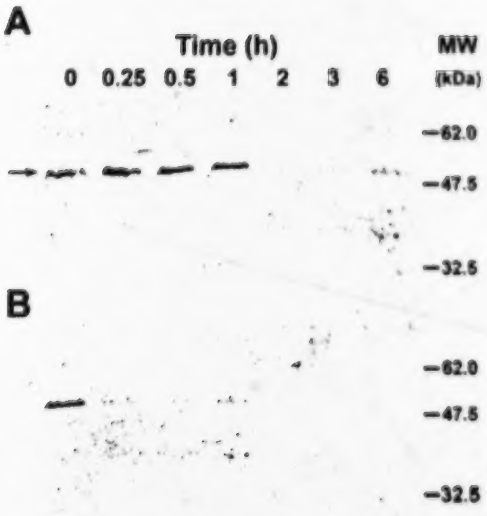
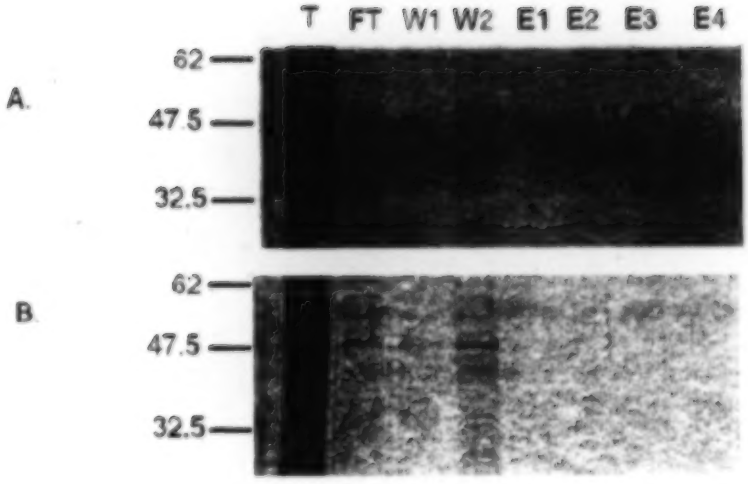

A
Time (h)
MW
0 0.25 0.5 1 2 3 6
(kDa)
62.0
47.5
32.5
B
62.0
47.5
32.5

Fig. 5.3. Phosphorylated proteins from 0.25 h cold shocked *Anabaena* cells.

Polypeptide analysis by 10 % SDS-PAGE of elution fractions from the PhosphoProtein purification column containing protein extract from 0.25 h cold shock cells, (A) without, and (B) with alkaline phosphatase treatment. The Coomassie blue stained gel is shown with the 34 kDa polypeptide indicated by one asterisk and the 53 kDa polypeptide indicated by two asterisks. T: total protein loaded on the column (0.1mg/ml), FT: flow through, W: wash, and E: elution.


T FT W1 W2 E1 E2 E3 E4
A.
62
47.5
32.5
B.
62
47.5
32.5

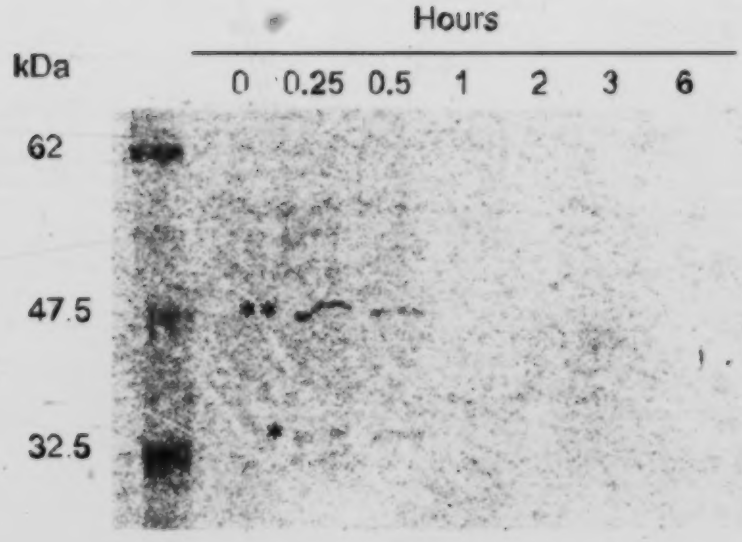
(not photographically reproducible) were detected in elution fractions 3 and 4 (Fig. 5.3A, lanes E3 and E4). Treatment of the protein extract with alkaline phosphatase prior to application to the IMAC column resulted in the absence of both proteins from all elution fractions (Fig 5.3B, lanes E1-E4), confirming the phosphorylation status of the 53 and 34 kDa proteins. IMAC was also utilized to investigate the *in vivo* phosphorylation profile in *Anabaena* cell free extracts at various times after cold stress. Coomassie blue stained SDS-PAGE analysis of proteins eluted from the phosphoprotein column indicates that phosphorylation of the 53 and 34 kDa polypeptides peaks after 0.25 hour of cold stress and then declines (Fig. 5.4). This *in vivo* profile of phosphorylation is similar to that obtained by *in vitro* phosphorylation of extracts obtained from cold stressed *Anabaena* (Fig. 5.1A).

5.2.4 Identification of purified cold stress phosphoproteins from *Anabaena*.

The 53 kDa and 34 kDa polypeptides were excised from a Coomassie stained SDS-PAGE gel, digested with trypsin, and subjected to MALDI-TOF analysis. MALDI-TOF analysis identified the 53 kDa polypeptide as EF-Tu and the 34 kDa polypeptide as ribosomal protein S2 (Rps2) from *Anabaena*. Since the predicted theoretical molecular mass of each protein, 44.8 kDa and 30.3 kDa respectively, was less than that estimated from SDS-PAGE gels, analysis for potential post-translational modifications was performed applying the approach reported by Wilkins *et al.* (1999). In addition to phosphorylation, two potential post-translational modifications, acetylation and methylation, were identified for EF-Tu using the protein modification program, FindMod. Modifications were putatively identified as including; acetylation on lysine-274 and trimethylation on arginine-29, aspartic acid-277 and 283, glutamic acid-278, and

Fig. 5.4. Cold shock phosphorylation profile examined by IMAC.

Polypeptide analysis by 10 % SDS-PAGE from the third elution fraction off the IMAC column of phosphorylated proteins over a 6 hr cold shock time course. The gel was stained with Coomassie blue and the indicated 53 kDa (one asterisk) and 34 kDa (two-asterisks) bands were excised for analysis by mass spectrometry.


Hours
kDa
0
0.25
0.5
1
2
3
6
62
47.5
32.5

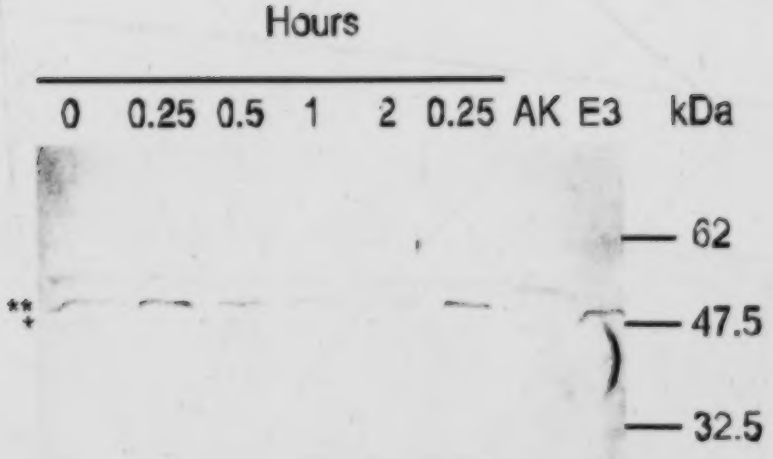
glutamine-284. Rps2 fragments analyzed also revealed potential post-translational modifications including phosphorylation at serine-28 and 34, tyrosine-30 and 32, or threonine-33 residues, and the presence of polyphosphate on lysine-152

5.2.5 The 53 kDa polypeptide is the phosphorylated form of EF-Tu.

To confirm the identity of the 53 kDa polypeptide Western blot analysis of protein extracts from cold stressed *Anabaena,* was performed with anti-EF-Tu antisera. The antisera cross-reacted with polypeptides of molecular masses of 45 kDa and 53 kDa (Fig. 5.5). In support of this observation, Huang *et al.* (2002), have reported that EF-Tu identified from *Synechosystis* sp. strain PCC 6804 has a theoretical molecular weight of 43.7 kDa; however, it was identified among plasma membrane proteins with an experimental molecular weight of 54 kDa which is in agreement with our findings. Accumulation of the 53 kDa polypeptide peaked within 0.25 h of cold shock and then decreased to background levels (Fig 5.5, two asterisks), while the level of the 45 kDa polypeptide remained relatively constant (Fig 5.5, one asterisk). Treatment of the cold stress cell free extract with alkaline phosphatase resulted in the disappearance of the 53 kDa polypeptide with a concomitant increase in the accumulation of the 45 kDa polypeptide (Fig. 5.5, lane AP), suggesting that the 53 kDa peptide represents the phosphorylated form of EF-Tu. This conclusion is verified by cross-reaction of the 53 kDa polypeptide, eluted from the phosphoprotein column, with the anti-EF-Tu antibody (Fig. 5.5, lane E3).

Fig. 5.5. EF-Tu profile during cold shock.

Western blot analysis of cell free protein extract from a 2 h time course of cold shocked *Anabaena* at the indicated times. AK: 0.25 h cold shocked cell free protein extract after treatment with alkaline phosphatase, E3: third elution fraction from the PhosphoProtein purification column of protein extract from 0.25 h cold shock cells. The blot was probed with anti-EF-Tu antiserum. Molecular mass of polypeptides are indicated by solid lines, 45 and 53 kDa are indicated by a • and ••, respectively.


Hours
0
0.25
0.5
1
2
0.25
AK
E3
kDa
62
47.5
32.5

5.3 Discussion

A primary physiological response of prokaryotes to decreased growth temperature is a reduction in general mRNA translation superimposed upon selective translation of cold shock mRNAs. The ribosome is a primary target of the cold shock response, becoming cold adapted through association with a range of *de novo* synthesized CSPs. The cold-adapted ribosome can presumably function efficiently and communicate mRNA selection at reduced temperature. The fact that a significant number of CSPs are translation IFs (IF1, IF2, and IF3) and ribosome associated (RbfA, CspA, CspB and Dead/CsdA) indicate that the ribosome itself is a primary target of the cellular response to cold shock and becomes cold adapted in response to cold shock (Gualerzi and Pon, 1990; Gualerzi *et al*., 2003; Giuliodori *et al*., 2004). However, the assumption that the ribosome is becoming cold adapted could be also attributed to post-translational modification where ribosomal and/or ribosome associated proteins are among the downstream targets of the cold signal transduction pathway. Therefore we have investigated the phosphorylation profile of cold shock in an attempt to identify downstream target(s) of the signal transduction pathway related to the inhibition of translation. Analysis of the cold-induced profile of phosphorylated proteins in the photosynthetic cyanobacterium, *Anabaena*, detected two proteins which are transiently hyperphosphorylated within 15 minutes of a temperature downshift. The proteins were purified by phospho-affinity chromatography and identified as the translation factors EF-Tu and ribosomal protein S2 (Rps2). Discrepancies between the theoretical and observed molecular mass of each protein, coupled with computer model prediction of post-translational modifications, are consistent with the post-translational modification of both EF-Tu and Rps2 by

phosphorylation, methylation, acetylation, or polyphosphate addition during cold stress. Information indicating post-translational modification of ribosomal proteins in response to cold stress is rare. Rps6 is hyperphosphorylated in response to cold stress in maize, where it has been proposed to contribute to the differential translation of ribosomal protein mRNAs (Williams *et al.*, 2003). Expression of two genes encoding Rps2-kinases is also enhanced in response to cold stress in *Arabidopsis*, thereby linking cold stress with a signal transduction pathway a target of which is a ribosomal protein (Mizoguchi *et al.*, 1995).

EF-Tu and Rps2 phosphorylation at reduced growth temperature may be involved in the reduction in general translation observed in response to cold stress. EF-Tu functions in the elongation phase of translation, each peptidylation reaction requiring an EF-Tu-GTP-aminoacyl tRNA ternary complex which provides an amino acid to the ribosomal A-site. It is well established that EF-Tu phosphorylation inhibits ternary complex formation, thereby reducing the rate of translation by inhibiting elongation as a result of the lack of activated EF-Tu complexes (Alexander *et al.*, 1995). Therefore, the expected physiological consequence of EF-Tu phosphorylation in *Anabaena* during cold stress would be a reduction in translation efficiency. Phosphorylation also protects *E. coli* EF-Tu from proteolytic cleavage (Lippman *et al.*, 1993), making it available for use once adaptation to cold stress has occurred. *E. coli* EF-Tu is phosphorylated on Thr382 a residue which is conserved in *Anabaena*. Mass spectrometry analysis also indicated that EF-Tu, in addition to phosphorylation, may also be post-translationally modified by methylation and acetylation in *Anabaena*. Evidence for the importance of protein methylation with respect to the regulation of translation has been reported. The *in vivo*

methylation of EF-Tu in *E. coli* prolongs the selection of an incoming ternary complex, thereby enhancing translational accuracy while decreasing the rate of protein synthesis (Van Noort *et al.*, 1986). In this capacity, EF-Tu methylation would also be expected to reduce the rate of translation while enhancing ribosomal proof reading fidelity during cold shock in *Anabaena*.

Similar to EF-Tu, Rps2 was also rapidly and transiently hyperphosphorylated in response to cold stress in *Anabaena*. Relatively little is known regarding the expression or regulation of Rps2 function in prokaryotes. Rps2 is required for the recruitment of Rps1 to the 30S ribosomal subunit during translation initiation, the appropriate interaction of Rps1 with Rps2 being required for translation in prokaryotes (Moll *et al.*, 2002). Rps1 is thought to mediate proper alignment of the mRNA start site on the 30S subunit association through the destabilization of secondary structures occurring at mRNAs 5′ UTR (de Smit and van Duin, 1994). Translation of highly structured mRNAs (van Dieijen *et al.*, 1995) but also the bulk mRNA translation is also Rps1 dependent (Sørensen *et al.*, 1998). Rps2 is an essential gene in yeast (Giaever *et al.*, 2002) and is known to be phosphorylated in yeast and mammalian cells (Jakiubowica, 1985; Beaud *et al.*, 1994). In HeLa cells, Rps2 is phosphorylated by a ribosome-associated kinase in response to vaccinia virus infection (Beaud *et al.*, 1994), but its phosphorylation level remains unchanged in response to hyperthermal conditions (Jakiubowica, 1985). While phosphorylation of translation initiation factors in response to abiotic stress is well known to regulate translation initiation in eukaryotes (Sheikh and Fornace, 1999), similar regulation in prokaryotes is lacking. It is tempting to speculate that Rps2 phosphorylation

inhibits interaction with Rps1. The lack of Rps1 association with the 30S subunit would lead to an inhibition in translation initiation.

Analysis of the mass spectrometry data with FindMod indicated that Rps2 was also post-translationally modified by the addition of inorganic polyphosphate. Inorganic polyphosphate is known to accumulate in response to a range of stress conditions, including amino acid starvation, osmotic stress, and nutritional limitations such as depletion of phosphate and nitrogen (Kuroda *et al.*, 1997; Ault-Riché *et al.*, 1998). The association of ribosomal proteins Rps2, RpL9, and RpL13 with inorganic polyphosphate enhances their degradation by the ATP-dependent protease, Lon, in response to starvation (Kuroda *et al.*, 2001). A similar situation may exist in *Anabaena*, in which inorganic polyphosphate addition to Rps2 during cold stress may lead to Lon-dependent proteolysis and the concomitant reduction in Rps2 levels. Thus, the combination of two post-translational modifications, phosphorylation and addition of polyphosphate, may synergistically reduce the level of functional Rps2 and thus translation initiation capacity. Since Rps2 is required for Rps1 interaction with the ribosome, a reduction in Rps2 activity by post-translational modification(s), would reduce Rps1 association with the ribosome. Rps1 possesses RNA helix destabilizing activity believed to be required for mRNA binding to the 30S ribosomal subunit during translation initiation. It is interesting that an *E. coli rpsB* mutant, encoding Rps2, is complemented by overexpression of the RNA helicase, DeaD (Toone *et al.*, 1991). DeaD, also termed CsdA, is a CSP which is ribosome associated during cold stress (Jones *et al.*, 1996). DeaD/CsdA has been shown to restore interaction of both Rps1 and Rps2 with the ribosome in a $rpsB^{ts}$ mutant (Moll *et al.*, 2002). This suggests, that in *E. coli*, a deficiency in Rps2 and thus Rps1 association

with the ribosome can be complemented by the RNA unwinding activity provided by a cold inducible RNA helicase. *Anabaena* also possesses a cold inducible RNA helicase, CrhC (Chamot *et al*., 1999; Chamot and Owttrim., 2000) which exhibits RNA helicase activity (Yu and Owttrim, 2000). CrhC may therefore contribute to the selective translation of CSP mRNA by enhancing Rps2/Rps1 association with the cold adapted ribosome through modification of 16S rRNA secondary structure or an unknown aspect of ribosome biogenesis during cold stress in *Anabaena*.

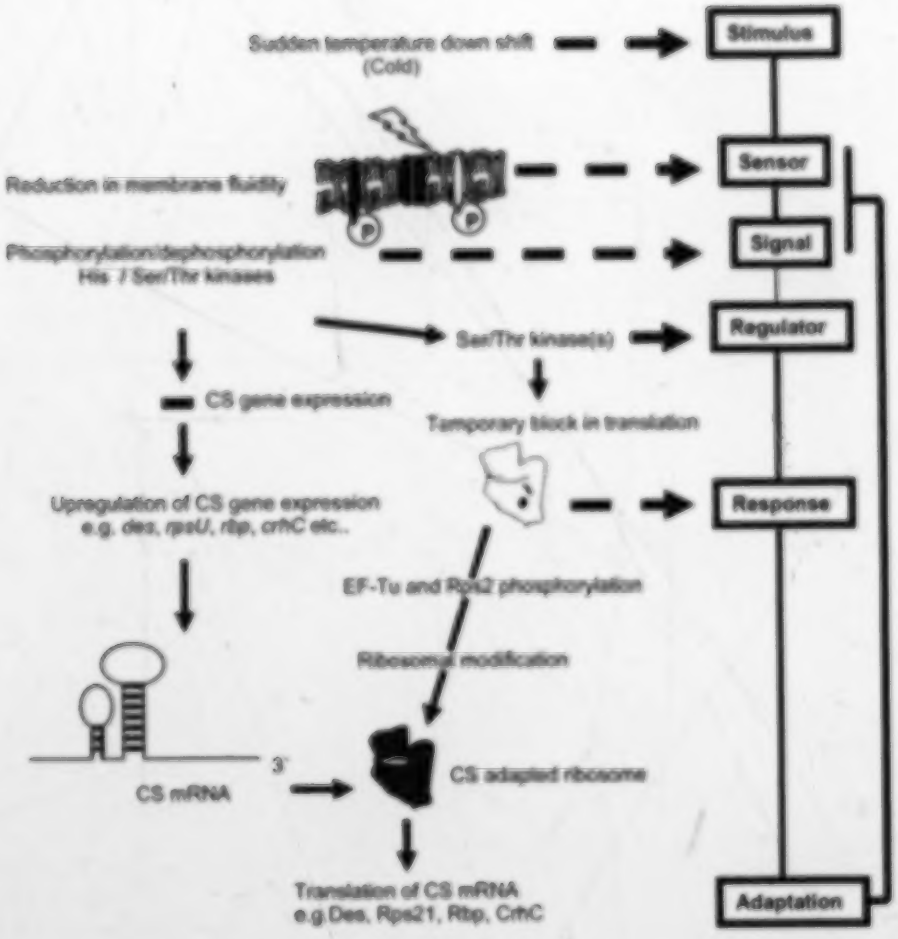
How are EF-Tu and Rps2 phosphorylated? Two-component signal transduction pathways involved in thermosensing of low temperature have been described in the cyanobacterium *Synechocystis* sp. strain PCC 6803 (Suzuki *et al*., 2000) and *Bacillus subtilis* (Aguilar *et al*., 2001). In both cases, a histidine kinase sensor and a transcriptional response regulator combine to regulate expression of a single lipid desaturase gene involved in alteration of membrane fluidity. The inability to detect additional CSP genes whose expression is regulated by these transcriptional regulators implicates the requirement for additional two-component signaling systems in low temperature sensing and signal transduction in these systems. This suggests the existence of a separate cold signal transduction pathway in bacteria regulating the expression of cold shock-induced genes. Recently a genomic analysis of protein kinases, protein phosphatases and two-component regulatory systems of the cyanobacterium *Anabaena* sp. strain PCC 7120 resulted in the identification of 52 genes encoding serine/threonine kinases (Wang *et al*., 2002). These results suggest that the cold signal transduction pathway may include a serine/threonine protein kinase response regulator whose targets include ribosome-associated proteins. Our results confirm this proposal, indicating that cold temperature

signal transduction in *Anabaena* involves a threonine kinase response regulator which involves threonine phosphorylation of EF-Tu post-translationally.

We propose a cold signal transduction model in *Anabaena* involving modification of ribosomal proteins by Ser/Thr kinase phosphorylation (Fig 5.6). Post-translational alteration of EF-Tu and Rps2, by phosphorylation and/or methylation, implicates two new factors which facilitate formation of a cold adapted ribosome. EF-Tu and Rps2 phosphorylation by a serine/threonine kinase could reduce the efficiency of translation initiation and elongation, respectively.

The results presented here provide insight into the mechanisms by which translation is down-regulated by decreases in the occupancy of the ribosomal A-site by aminoacyl-tRNA (aa-tRNA) and the efficiency of translation initiation in response to cold shock (Graumann and Marahiel, 1996). The post-translational modification of EF-Tu and Rps2 in response to cold stress are reminiscent of the ribosome filter hypothesis proposed as a translational regulatory mechanism in eukaryotes (Mauro and Edelman, 2002). In this context, cold adapted ribosomes, altered by interaction with accessory factors, may selectively enhance translation of cold shock mRNAs. In conclusion, two new ribosomal proteins, EF-Tu and Rps2, whose activity and not transcription is post-translationally altered in response to cold stress have been identified. The resulting reduction in activity and ribosomal interaction would contribute to the formation of a cold adapted ribosome, resulting in a reduction in general translation and potentially aiding selective translation of CSP mRNA.

FIG. 5.6. Proposed model for cold shock signal transduction pathway in *Anabaena* (see text for details).


Sudden temperature down shift
(Cold)
Stimulus
Reduction in membrane fluidity
Sensor
P
P
Phosphorylation/dephosphorylation
His / Ser/Thr kinases
Signal
Ser/Thr kinase(s)
Regulator
CS gene expression
Temporary block in translation
Upregulation of CS gene expression
e.g. des, rpsU, rbp, crhC etc..
Response
EF-Tu and RpsL2 phosphorylation
Ribosomal modification
3'
CS mRNA
CS adapted ribosome
Translation of CS mRNA
e.g.Des, Rps21, Rbp, CrhC
Adaptation

Chapter 6

Discussion

6. Insights towards understanding the perception of cold shock and cellular response

The major physiological effects of cold shock include a decrease in membrane fluidity and protein synthesis. Therefore, adaptation to cold shock requires the adjustment of membrane fluidity, and bypassing a translational block at the ribosome.

6.1. The state of the ribosome in relation to protein synthesis in cold shock

The involvement of the ribosome in cold shock response was initially reported by Das and Goldstein (1968) and Friedman *et al.*, (1969), where they found that an unknown component(s) of the ribosomal fraction of the bacterial cell is responsible for the arrest of translation at low temperature. Also, the ribosome was proposed to function as a thermosensor in bacteria, where its state (occupancy of the ribosomal A-site) signals for the induction of temperature responses (Van Boghelen and Niedhardt 1990). Moreover, it has been anticipated that the ribosome becomes temporarily non-functional for translating cellular mRNAs resulting in a growth lag period (Jones and Inouye, 1994). During this lag period, translation initiation of the majority of cellular mRNAs is significantly reduced while translation of mRNAs encoding CSPs is maintained. This discrepancy must be resolved in any model describing the physiological response to cold shock.

Although no direct data is presented in this thesis, our working model assumes that secondary structure within the 5′ UTRs of cold shock mRNAs is involved in regulation of gene expression in response to cold shock. Specific recognition and binding and subsequent active ATP-dependent unwinding of these structures by CrhC has the potential to promote efficient translation initiation of cold shock mRNAs. This model still does not address the mechanism by which cold shock leads to inhibition of translation

initiation on the majority of mRNAs. However, it can be envisioned that cold shock could result in translation inhibition at either translation initiation or elongation. This general inhibition would then have to be overcome by altering ribosomal components converting it into a cold adapted state. The cold adapted ribosome would have the ability to bypass the mechanisms leading to the general inhibition of translation and also allowing the selective translation of cold shock mRNA. In support of this proposal, several translational machinery components have been reported to be cold-induced including translation initiation factors (IF1, IF2, and IF3) (Giuliodori *et al.*, 2004), ribosomal proteins (Rps1B, and Rps21) (El-Fahmawi and Owttrim 2005; Sato, 1994), and the ribosomal associated protein A (RbfA). A portion of the material presented in this thesis also supports the production of a cold shock adapted ribosome which is achieved by post-translational modification events in the form of increased phosphorylation of ribosomal protein S2 and the EF-Tu. This allows extension of the model to include mechanism(s) by which translation is inhibited during cold shock.

The ribosome maintains high protein synthesis fidelity under all stress conditions including cold shock. This is most likely achieved by conversion of the ribosome to a cold adapted state. Evidence presented in this thesis indicates a component of the ribosome adaptation involves post-translational modifications including phosphorylation of ribosomal protein(s) involved in translation such as Rps2 and EF-Tu. Rps2 is required for the recruitment of Rps1 at the translation initiation step on the 30S subunit of the ribosome. The recruitment of Rps1 to the 30S subunit is an essential step in translation initiation. Since the major metabolic block during cold shock is proposed to occur at translation initiation, the hyper-phosphorylation of Rps2 can be hypothesized to result in

a conformational change that will not allow it to bind to the 30S subunit. This would prevent Rps1 binding, resulting in a temporary inhibition in translation initiation in response to cold shock. The other concurrent modification observed in this thesis is the hyper-phosphorylation of EF-Tu. EF-Tu phosphorylation has been shown to prevent ternary complex formation (EF-Tu-GTP-aatRNA) (Alexander *et al.*, 1995), thereby inhibiting protein synthesis at the elongation step.

The elongation step has been proposed to be a detrimental step for regulating the speed of translation during thermal shock, which appears to be dependent upon the occupancy of the A site at the ribosome. Cold shock results in high concentration of charged tRNA leading to the blocking of the A site, and thus inhibition of the elongation step (Graumann and Marahiel, 1996). Furthermore, a second cold-induced protein from *E. coli*, protein Y, has recently been shown to specifically inhibit translation by blocking the peptidyl- and aminoacyl-tRNA sites thereby inhibiting translation initiation and elongation during cold shock. Therefore, translation inhibition by protein Y through the blocking of aminoacyl-tRNA binding to the A-site at the elongation stage is in agreement with the proposal that the occupation of the A-site triggers cold and heat response in bacteria (Agafonov *et al.*, 2001; Vila-Sanjurjo *et al.*, 2004). These findings position the translation initiation and elongation as major targets for the cold shock response and adaptation.

6.2. The cold shock signal transduction pathway

The existing data may explain some missing parts in the jigsaw of cold shock response and adaptation, which allows expansion of the cold shock response and

adaptation network. Here, I propose that the cold shock signal is perceived as a change in the plasma membrane fluidity (reduced membrane fluidity), which activates and/or triggers a signal transduction pathway conveying the cold shock signal. This cold signal transduction pathway relays the signal by a specific phosphorylation/dephosphorylation of downstream protein targets by a network of kinases and phosphatases. The phosphorylation/dephosphorylation state of these response regulator targets alters cellular function thereby producing the characteristic physiological responses observed during cold shock. This occurs through the regulation of cold shock gene expression, and secondly, by direct modulation of the CSPs function involved in the regulation and maintenance of vital cellular processes such as protein synthesis and membrane fluidity during cold shock.

The involvement of a two-component signal transduction system has been described as a cold thermosensor in *B. subtilis* (Aguilar *et al.*, 2001; Cybulski *et al.*, 2004). This proposed sensor is composed of a sensor kinase, DesK, and a response regulator DesR, responsible for the cold induction of the *des* genes coding for Δ5-lipid desaturase, which add a double bond(s) into the hydrocarbon chain of membrane fatty acids. The desaturation of membrane fatty acids will reverse the reduction in membrane fluidity resulting from cold shock to normal fluidity. A similar signaling system has also been identified in the cyanobacterium *Synechosystis*, where Hik33 (sensor kinase) and Hik19 (response regulator) were necessary for low temperature induction of *desB* expression (Suzuki *et al.*, 2000 and 2001). However, their involvement in the coordinated cold shock response was restricted to a limited number of cold shock-induced genes including *desB*, with insignificant effect on the expression of two other cold-induced

genes, *desA* and *crh* (cold-induced RNA helicase) (Suzuki *et al.*, 2001; Mikami *et al*, 2002; Marin *et al.*, 2003). This data suggests the existence of additional signal transduction pathway(s) involved in the regulation of gene expression in response to cold shock. The lack of a single two component system in the regulation of environmental stress is becoming increasingly common in prokaryotes including cyanobacteria. For example salt and osmotic shock appear to have numerous two component systems involved in their signal transduction (Suzuki *et al.*, 2001; Mikami *et al*, 2002; Marin *et al.*, 2003). These results lead me to propose that the actual temperature sensor in *Synechocystis* is not the Hik33 kinase but remains to be identified. One suggestion for the cold sensor comes from eukaryotic systems: in plants it was shown that the signal of temperature change is perceived as alterations in membrane fluidity in response to cold shock (i.e. membrane rigidification) or heat shock (i.e. an increase in membrane fluidity) that translate the signal via the cytoskeleton, Ca^{2+} fluxes, and calcium dependent protein kinases (Sangwan *et al.*, 2002). Calcium levels in cyanobacteria are also mobilized in response to temperature change (Torrecilla *et al.*, 2000). Similar to plants and mammalian cells, Ca^{+2} in cyanobacteria may act as a messenger in the signal transduction of cold shock, where calcium activates Ca^{+2} dependent kinases (serine/threonine and tyrosine), allowing downstream cold shock signal transduction (Hirt, 2000). An example of a calcium activated kinase is eEF2 kinase which is responsible for the phosphorylation of eukaryotic elongation factor (eEF) 2. The phosphorylation of eEF2 results in the inhibition of protein synthesis at translation elongation (Sangwan *et al.*, 2002; Browne *et al.*, 2004). Interestingly, the increased phosphorylation of EF-Tu and Rps2 on a serine/threonine residue in response to cold shock in *Anabaena*, implicates the

involvement of a serine/threonine kinase(s) in the cold shock signal transduction pathway. This kinase(s) may have multiple roles in the cold shock response, including: (i) directly modulating protein translation by phosphorylating EF-Tu and Rps2, and (ii) by phosphorylation of response regulator protein(s) involved in regulating cold shock gene expression. In support of this idea, the lag period observed in response to cold shock may be required to allow for the expression of cold shock genes and to adjust the ribosome to become cold adapted allowing the translation of cold shock gene products.

The temporary increase in the phosphorylation of Rps2 and the *de novo* synthesis of Rps1B further suggest that the ribosome undergoes modifications in order to adapt to cold shock. Such modifications will allow for the revision of translational efficiency by delaying the initiation step, allowing the ribosome to accommodate the translation of the cold shock mRNA with highly structured 5′ UTRs. An example of such temporary delay in translation during cold shock is observed in the expression pattern of *crhC*, where a lag period of ≈ 15 min was observed between *crhC* mRNA accumulation and CrhC expression (Chamot *et al.*, 2000).

6.3. The correlation between protein synthesis and the functionality of the membrane during cold shock

The regulation and maintenance of an efficient protein synthesis and the recruitment of these proteins at the site of their function is essential and a priority to the cell for its wellbeing. However, during cold shock, membrane fluidity and integrity is compromised.

The plasma membrane is a highly specialized structure representing the first level of contact between the cell and the surrounding environment. This level of specialization is generated from its unique structure, which is composed of a phospholipid bilayer and proteins. The membrane associated proteins possess a vast array of functions including catalytic, metabolic, transport, cell division, integrity and support of the membrane in particular and the cell as a whole. Therefore any change in membrane fluidity may affect numerous enzymatic and transport processes making them compromised during cold shock (Han *et al.*, 2005). The *de novo* synthesis and insertion of membrane proteins involved in transport and/or membrane enzymatic processes into or across the membrane is essential for the cell to grow and divide. Therefore it is not surprising for CSPs to be localized at the ribosome and at the periphery of the cell (poles) in close proximity to the membrane only upon active transcription, thus coupling transcription to translation at the plasma membrane. These observations suggest that a mechanism may exist during cold shock for co-translational insertion of membrane proteins involving plasma membrane associated ribosomes for adjusting membrane fluidity (e.g. membrane associated desaturases, porin proteins) and maintaining membrane integrity and function including cell division, nutrient transport, protein translocation, and energy generation. Therefore, it is plausible to speculate that facilitating and/or maintaining of the synthesis of proteins required for adjusting the membrane fluidity and function is prioritized by the translation machinery as an essential step required for cold shock response and adaptation.

6.4. A model for cold shock response and adaptation from *Anabaena*

As described earlier, cold shock is sensed in mammalian and plant cells as a decrease in the fluidity of the plasma membrane through fluxes in Ca^{2+} levels, leading to the activation of calcium dependent protein kinases (e.g. eEF2 kinase) (Hirt, 2000; Sangwan *et al.*, 2002; Browne *et al.*, 2004).

Likewise, in *Anabaena* the intracellular level of calcium increases in response to cold shock (Torrecilla *et al.*, 2000). Therefore, *Anabaena* may perceive the cold shock as a change in membrane fluidity relaying the signal via a change in Ca^{2+} fluxes that activates specific kinases and phosphatases resulting in the phosphorylation/dephosphorylation of protein(s) which directly affects potential function(s) including the selective and differential expression and/or repression of genes, and the modulation of cellular components such as the ribosome. Here I propose that among the potential kinase(s) involved in the cold shock signal transduction pathway in the cyanobacterium *Anabaena* is a serine/threonine kinase which is responsible for the temporary hyperphosphorylation of EF-Tu resulting in a block in translation at the elongation step, and Rps2 which may modulate translation initiation.

Other potential downstream targets of this serine/threonine kinase are response regulator proteins involved in regulating cold shock genes expression. In support of this proposal, preliminary data suggest that the expression of *crhC* appears to be regulated by the level of phosphorylation of a protein that is yet to be identified, which may represent the first example for gene regulation by phosphorylation in response to cold shock (Brown and Owttrim, unpublished observation).

Thus, a model for cold shock sensing and adaptation is proposed (Fig. 6.1). In this model, the cold signal is perceived as a change in membrane fluidity resulting in Ca^{+2} flux changes which triggers the cold shock signal transduction pathway involving a cascade of phosphorylation/dephosphorylation of down- stream targets by a network of kinase(s)/phosphatase(s) including serine/threonine kinase(s). Among the kinase(s) down-stream targets are the ribosome, and the regulatory DNA binding protein(s) where differential phosphorylation regulate the cold shock genes expression. In this model I propose that the first step towards a cold adapted ribosome involve an increased phosphorylation of EF-Tu. The consequence of the phosphorylation of EF-Tu will temporarily halt translation elongation, resulting in the clearing of the ribosome of any non-cold shock mRNA being translated at the moment of the cold shock. Therefore, the block in translation at the elongation step may represent the signal for the expression and recruitment of ribosomal proteins at the 30S subunit (e.g. Rps1B and Rps21) to modulate its translation machinery at the translation initiation step to accommodate the translation of cold shock mRNAs resulting in a cold shock adapted ribosome

Concurrently, the ribosome is further modified by an increase in the phosphorylation of Rps2 and the *de novo* expression of Rps1B. Rps2 is required for loading of Rps1 to the 30S subunit for translation to be initiated. Interestingly, the *Anabaena* genome has two Rps1 genes (*rps1A* and *rps1B*). The expression of Rps1A is constitutive, where Rps1B is cold induced. Therefore it is tempting to speculate that the phosphorylation of Rps2 will facilitate the recruitment of Rps1B to the 30S subunit, which can be an essential step for the selective loading of cold shock mRNA to the translation initiation complex of the cold adapted ribosome. However, this step may not

be enough to destabilize the thermodynamically stabilized long, highly structured 5′ UTRs of cold shock mRNAs. Therefore, active unwinding or destabilizing of secondary structures in 5′ UTRs will be required for efficient translation of cold shock mRNA. In this scenario, the 5′ to 3′ RNA helicase activity possessed by CrhC could potentially function to unwind secondary structure in the 5′ UTR of target mRNAs, allowing translation initiation to proceed (Yu and Owttrim, 2000). In support of this idea, several 30S ribosomal proteins co-purify with the cold-induced *E. coli* RNA helicase, CsdA, including Rps1 and Rps2 (Butland *et al.*, 2005). Furthermore, CrhC may also function at the elongation stage, where its mechanistic ability as an RNA helicase may allow it to knock out any RNA binding proteins that cause translation repression similar to the Y protein or Hfq. Despite that the mechanism by which this could be accomplished is not known, the co-purification of Hfq, the *ompA* mRNA translation repressor, with CSPs, CsdA, Rps1, Rps2, SecA, and EF-Tu may support the above mentioned proposal (Butland *et al.*, 2005).

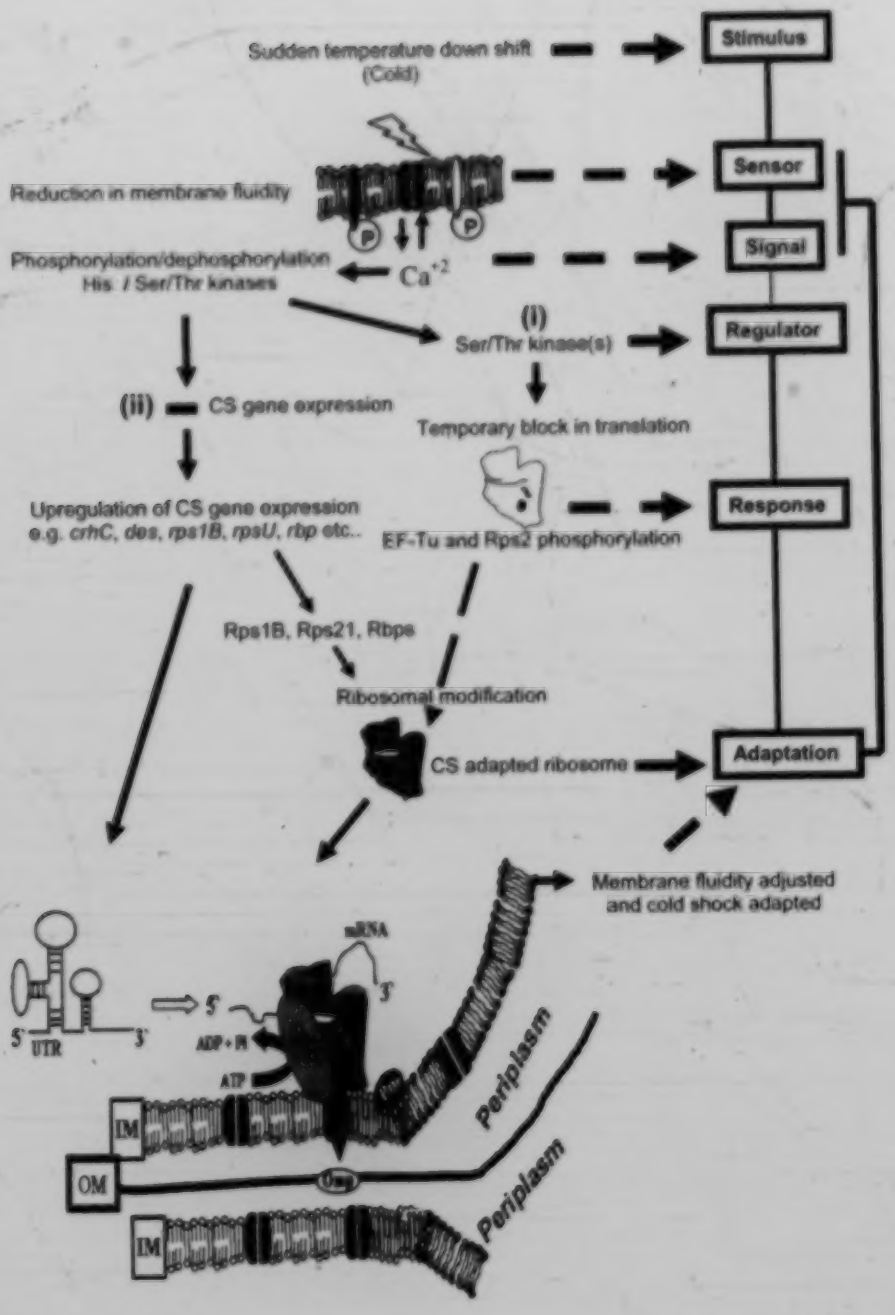
The asymmetrical distribution with polar bias of CrhC to the plasma membrane adds another interesting piece which must be included in any model describing cold shock response and adaptation. The combination of the results that CrhC co-immunoprecipitates with plasma and outer membrane proteins, and co-localizes with the ribosome at the plasma membrane makes it intriguing to speculate on its potential role during the cold shock response. Accordingly, I propose that CrhC functions in a complex involved in the co-transcriptional translation (transertion) of CSPs and/or essential proteins targeted to or across the plasma membrane (e.g. OMPs, ATP synthase α chain, nitrate transport protein, cytochrome F, desaturases etc…), and thus allows translation to

proceed and the resulting polypeptides to be directly inserted or transported across the plasma membrane. However, to fulfill this role CrhC has to be associated with the ribosome and the translocon at the plasma membrane in a manner similar to SecA. The co- and post-translational proteins translocation by the Sec-dependent pathway involves SecA, which possess an RNA helicase activity similar to CrhC (Facey and Kuhn, 2004; Vrontou and Economou, 2004; Karamanou *et al.*, 2005). In *E. coli*, Rps2, EF-Tu, Hfq, OmpA co-purified with SecA (Butland *et al.*, 2005), which may position SecA at the translation initiation and elongation. However, none of the CSPs were among the proteins co-purified with SecA (Butland *et al.*, 2005). Interestingly, evidence has been presented indicating that the Sec-dependent protein translocation process is cold sensitive (Pogliano and Beckwith, 1993). Therefore, a potential role for CrhC as a component of a cold adapted translocation apparatus functioning similar to SecA may exist, where CrhC functions in maintaining efficient and active protein secretion during cold shock. This will allow direct translocation and targeting of proteins into or across the plasma membrane required for membrane trafficking, cell-cell communication, cell division, environmental sensing, and other membrane related functions.

In summary, this model provides new avenues to cold shock research, where systematic disruption of Ser/Thr kinases will bring us closer to the identification of the cold shock signaling mechanism(s) and the networking of cold shock response including identification of downstream targets involved in the regulation of the expression of cold shock genes and translation machinery. Also, the identification of cold shock related modifications in the translational machinery will provide a better understanding of the mechanism(s) regulating protein synthesis during cold shock.

Fig. 6.1. A model for cold shock response and adaptation from *Anabaena*.

A cold shock stimulus is perceived as a change in membrane fluidity which results in an increase in Ca^{+2} influx. Calcium activates serine/threonine and histidine kinases. These kinases will convey a sequence of phosphorylation/dephosphorylation events resulting in (i) phosphorylation of EF-Tu and Rps2 resulting in a cold adapted ribosome, and (ii) the upregulation of cold shock genes, whose protein products will participate in alleviating the block in translation during cold shock (e.g. Rps1B, Rps21, Rbps, and CrhC) and the adjustment of membrane fluidity (desaturases). The cold adapted ribosome is ready for the translation of cold shock mRNA, where CrhC participate in a complex involved in the co-transcriptional translation (transertion) of CSPs and/or essential proteins targeted to or across the plasma membrane (e.g. OMPs, ATP synthase α chain, nitrate transport protein, cytochrome F, desaturases etc...) required for the cold shock adaptation of the plasma membrane.


Sudden temperature down shift
(Cold)
Stimulus
Reduction in membrane fluidity
Sensor
Phosphorylation/dephosphorylation
His / Ser/Thr kinases
Ca+2
Signal
(i)
Ser/Thr kinase(s)
Regulator
(ii)
CS gene expression
Temporary block in translation
Upregulation of CS gene expression
e.g. crhC, des, rps1B, rpsU, rbp etc..
EF-Tu and Rps2 phosphorylation
Response
Rps1B, Rps21, Rbpa
Ribosomal modification
CS adapted ribosome
Adaptation
Membrane fluidity adjusted
and cold shock adapted
mRNA
5'
3'
UTR
ADP + Pi
ATP
IM
OM
Periplasm
Periplasm
IM

6.5. Insights towards future research

The findings of this thesis not only provide insights towards understanding cold shock response and adaptation but also provide directions towards future research. Understanding the function of CrhC is still one of the main challenges in this research. The isolation of proteins interacting *in vivo* with CrhC will provide better understanding of its function during cold shock. Furthermore, the immunogold particles localization are in close proximity to the cytoplasm and appears to be possibly caught in a channel or a pore being involved in channeling proteins or RNA(s) involved in cell-cell communication, which is worth investigating further.

Another challenge in understanding the cold shock response is the identification of a serine/threonine kinase involved in the cold shock signal transduction pathway of which EF-Tu and rps2 are down stream targets.

6.5.1 *In vivo* Biotinylation of CrhC

Recently, biotinylation of protein tags has been utilized in the recovery and characterization of protein complexes (Penalva *et al.*, 2004). This procedure can be utilized to isolate proteins interacting *in vivo* with CrhC. This approach utilizes the co-transformation of cells with two plasmids in a *crhC* null mutant at normal growing temperature (30°C). One expresses CrhC tagged with a biotin acceptor peptide (BAP), a protease recognition site is placed between CrhC and BAP, the other plasmid expresses a biotin labeling enzyme, BirA. The biotinylation of the BAP tag only occurs in the presence of BirA upon the addition of exogenous biotin. The biotinylated CrhC with proteins interacting with it can be recovered by incubating the cell lysate with streptavidin-spharose beads. Proteins interacting with CrhC can be recovered by a

protease treatment that will cleave specifically between CrhC and the BAP. The recovered CrhC-protein(s) complex can be analyzed by SDS-PAGE, where the identity of different proteins in the complex can be identified by mass spectrometry analysis.

6.5.2 CrhC localization at the membrane

The possibility that CrhC might be involved in a secretion pathway at the poles within a filament for small RNA responsible for gene regulation at the translation initiation level in response to cold shock can be investigated by immunoprecipitating CrhC and identifying target RNA. After cold shock, cells can fixed in formaldehyde to maintain protein-RNA interaction, by utilizing anti CrhC antibodies, CrhC-RNA can be enriched and isolated. Follwed by RT-PCR, CrhC RNA substrates can be identified. The identification of RNA(s) among CrhC substrates will suggest its involvement in RNA transport between cells within a filament thereby functioning as a form of cell-cell communication.

6.5.3 Identification of a serine/threonine kinase involved in the cold shock signal transduction pathway

The systematic disruption of serine/threonine kinases will bring us closer to the identification of the cold shock signaling mechanism(s). The effect of the disruption of each of the 52 serine/threonine can be examined in relation of the loss of the hyperphosphorylation of EF-Tu and rps2 during cold shock will help identify the serine/threonine kinase responsible for such an event.

CHAPTER 7

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